EXPRESSION REGULATION OF ENDOMETRIAL ION CHANNELS BY STEROID HORMONES

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Declaration

This is to declare that the work presented in this thesis is my own and has not been submitted to this or any other institution for any degree, diploma or other qualification.

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Abstract

The endometrium and its absorptive and secretory activities are under constant influence of ovarian hormones (estrogen and progesterone), thereby providing an optimal fluid microenvironment for various reproductive events. Previous studies have demonstrated substantial amiloride-sensitive basal current, mediated by epithelial Na^+ channel (ENaC), and stimuli-activated I_{SC} mediated by cystic fibrosis transmembrance conductance regulator (CFTR) in mouse endometrial epithelial cells. The present studies further investigated the effect of ovarian hormones on CFTR and ENaC expression and function in mouse endometrial epithelial cells *in vivo* and *in vitro* by capillary electrophoresis-laser induced florescence (CE-LIF), semi-quantitative reverse transcription-polymerase chain reaction (RT-PCR) analysis and short-circuit current (I_{SC}) technique.

The level of CFTR expression in mouse uterus was found to vary throughout the estrus cycle. CFTR was detected maximally at proestrus and estrus which coincide with high plasma concentration of estrogen, while all ENaC subunits (α, β and γ) were abundantly detected at metestrus and diestrus when progesterone is at a maximum. Treatment of the cultured mouse endometrial epithelial cells with 0.01μM 17β-estradiol resulted in an increase in the forskolin-induced I_{SC}, presumably mediated by CFTR, from $5.1 \pm 0.1 \mu A/cm^2$ (control) to $8.3 \pm 0.7 \mu A/cm^2$ ($P<0.05$, $n=6$). In ovariectomized mice, injection of 17β-estradiol increased CFTR mRNA level and decreased γ-ENaC mRNA level, while the treatment of progesterone decreased CFTR

and increased α-ENaC and γ-ENaC mRNA level. Our results indicated that estrogen increased CFTR expression both *in vitro* and *in vivo*, while progesterone upregulated ENaC and downregulated CFTR expression *in vivo*.

Culture conditions can be varied by various factors including phenol red, a pH indicator widely used in growth medium and steroid hormones, present in the supplement fetal bovine serum (FBS). The present study examined the effect of phenol red and steroid hormones contained in FBS, on primary cultured endometrial epithelial cells by monitoring ion channel activities using short-circuit current technique. When compared to the results obtained with normal medium supplemented with regular FBS, the forskolin-stimulated I_{SC} obtained in phenol red free medium was significantly reduced, from 16.95 ± 1.53 $\mu A/cm^2$ (control) to 9.72 ± 0.89 $\mu A/cm^2$ ($P<0.05$). The forskolin-activated I_{SC} was further attenuated to 5.29 ± 0.46 $\mu A/cm^2$ when culture medium was supplemented with charcoal/dextran treated FBS where steroid hormones were deprived.

It is well known that ENaC expression could be regulated by aldosterone, a steroid hormone which controls the rate of transepithelial Na^+ absorption in a variety of epithelia. The present study further investigated the effect of aldosterone and Matrigel, a coating substrate for cell culture, which resembles the extracellular matrix facilitating rapid epithelial reconstitution and differentiation on endometrial epithelial cells by I_{SC} technique and RT-PCR analysis. Our results showed an increase in γ ENaC mRNA expression in both aldosterone treated and Matrigel-coated membrane, but not α and β subunits.

In order to study a possible regulatory role of aldosterone *in vivo*, the mRNA expression of uterine CFTR and ENaC was examined throughout the estrus cycle with mice fed with a low sodium diet for two weeks. The results showed that when fed with a low Na^+ diet, which was expected to elevate the circulating aldosterone, the cyclic characteristics of expression pattern for all ENaC subunits remained unchanged, but the maximal level of α and γENaC expression was considerably elevated, 121.9 % and 283.3 %, respectively; indicating a predominant role of α and γENaC subunit in regulating ENaC activity and thus the rate of Na^+ absorption across mouse endometrium. However, the mRNA expression of CFTR was suppressed throughout estrus cycle suggesting that expression of CFTR is also under the influence of aldosterone.

In Summary, the present data showed that CFTR and ENaC were differentially regulated by estrogen and progesterone both *in vivo* and *in vitro*. The present results also indicate that the expression of endometrial ion channels is also under the influence of aldosterone, suggesting these ion channels also contribute to overall body electrolyte and fluid balance. It is hoped that the result of present study may shed light on the understandings of the hormonal regulation of ion channels, and thus electrolyte and fluid transport, in the endometrial epithelium and body fluid homeostasis. The results of present studies may provide grounds for the development of contraceptives and possible new treatment for infertility.

論文撮要

子宮內膜及其吸收和分泌活動受到卵巢激素（雌激素和孕激素）的持續影響，從而爲各種生殖過程提供最佳的液體微環境。以前的研究已經證明小鼠子宮內膜上皮細胞存在的 amiloride 敏感的基礎電流很可能由上皮細胞鈉通道 (ENaC) 介導，而受到刺激時短路電流則是由囊性纖維變性跨電導調控器 (CFTR) 所介導的。本研究利用毛細管電泳鐳射誘發螢光技術(體內)、競爭性逆轉錄聚合酶鏈式反應（體內）分析和短路電流技術（體外），進一步考察了卵巢激素對子宮內膜細胞 CFTR 和 ENaC 的表達和功能方面的作用。

研究中發現 CFTR 的表達水平隨月經周期變化，檢測到 CFTR 表達最多時爲動情前期和動情期，與期間血漿的高雌激素濃度一致。而 ENaC 的所有亞單位 (α, β, 和 γ) 在動情後期和動情間期被大量地檢測到，此時孕激素的濃度最高。用 0.01 μM 的 17β-雌激素處理小鼠子宮內膜上皮細胞，其 forskolin 引起的短路電流從 5.1±0.1 μA/cm^2 (對照) 增加到 8.3 ±0.7 μA/cm^2 ($p<0.05$, $n=6$)。將去除卵巢的小鼠注射 17β-雌激素可增高 CFTR 的 mRNA 表達和減低 γ-ENaC 的 mRNA 表達。而注射孕激素，則減低 CFTR 的表達和增高 α-ENaC 和 γ-ENaC 的 mRNA 表達。我們的結果提示，雌激素不論在體內或體外都能增加 CFTR 的表達，而孕激素在體內則起上調 ENaC 以及下調 CFTR 的作用。

細胞培養條件可受多種因素影響，包括廣泛用於培養液的酚紅(一種 pH 指示劑)和類固醇激素(存在於補充的小牛血清中)。我們利用短路電流技術檢測離子通道活動，進一步研究了酚紅和類固醇激素對原代培養的子宮內膜上皮細胞的影響。與使用補充小牛血清的正常培養液相比較，在使用無酚紅的培養液時，

forskolin 刺激引起的短路電流(由 CFTR 所介導)從 16.95±1.53 μA/cm² (對照)大幅度減小到 9.27±0.89 μA/cm² ($p<0.05$)。當培養液補充小牛血清以木炭/右旋糖苷處理過(類固醇激素被去除)後 forskolin 啟動的短路電流進一步減小到 5.29±0.46 μA/cm²。

人們已經知道 ENaC 的表達受醛甾酮的調控，醛甾酮爲一類固醇，能調控多種上皮細胞的跨上皮鈉吸收率。我們利用短路電流技術和 RT-PCR 分析，進一步研究了醛甾酮和 Matrigel(一種用於促進上皮快速重建和分化的細胞外基質的類似物)對子宮內膜上皮細胞的影響。結果顯示，在經醛甾酮處理或支撐膜塗有 Matrigel 時。γ亞單位的 mRNA 表達明顯增加，而α和β亞單位的 mRNA 水平則無變化。爲探究醛甾酮對子宮內膜可能的調控作用，用低鈉食物飼養小鼠，期望能提高血循環中的醛甾酮,並在整個月經周期中檢測 CFTR 和 ENaC 的 mRNA 表達。結果顯示，採用低鈉食物飼養時，ENaC 所有亞單位表達的周期特徵保持不變。但α和γENaC 亞單位的最高表達水平則分別顯著地增大了 121.9% 和 283.3%。我們的資料提示γ-ENaC 亞單位在調控 ENaC 活動中所起的主導作用和由其所產生的跨小鼠子宮內膜吸收率。然而，CFTR 的 mRNA 表達在整個月經周期中都被調低。顯示 CFTR 的表達也受醛甾酮影響。

我們的資料顯示 CFTR 和 ENaC 在體內和體外都由雌激素和孕激素以及醛甾酮進行差異的調控。實驗的結果還提示了γ-ENaC 亞單位在決定 ENaC 的活動方面所起的重要作用。子宮離子通道的表達也受醛甾酮影響，提示這些離子通道也參與調節身體整體的電解質和體液平衡。希望本研究的結果對理解子宮內膜上皮中離子通道的激素調控、電解質和液體轉運開闢新的視野。本研究的結果可能爲開發避孕藥物和治療不孕提供新的線索。

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This thesis is dedicated to my family.

Table of Content

List of Publications

Part of the results presented in this thesis have been submitted for publication:

1. Distribution of Epithelial Na^+ Channel (ENaC) Subunits and Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in murine Female Reproductive Tract . (2001)

   Journal of Membrane Biology, submitted

   (with Chan LN, LG Rochelle, RC Boucher, Chan HC)

2. Culture condition on expression and function of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in Mouse Endometrial Epithelial Cells. (2001)

   Cell BiologyInternational, in press

   (with Chan HC, Chan LN)

3. Regulation of epithelial Na^+ channel activity by differential expression of its γ subunit in mouse endometrial epithelium. (2001)

   Japanese Journal of Physiology, in press

   (with Wang XF, JPS Yuen, RR Fiscus, Chan HC)

Related publication:

1. Suppression of CFTR-mediated Cl^- secretion by enhanced expression of epithelial Na^+ channels in mouse endometrial epithelium. (2000)

   Biochemical Biophysical Research Communications, vol. 276(1):40-4.

   (with Chan LN, Wang XF, Liu CQ, Chan HC.)

2. Regulation of Na^+ absorption and Cl^- secretion in the endometrium: switching mechanisms. (2000)

Journal of Korean Medical Science, vol. 15 Suppl:S34-5.

(with Chan HC, Chan LN, Wang XF, So SC.)

3. Pyrimidinoceptors-mediated activation of Ca^{2+}-dependent Cl^- conductance in mouse endometrial epithelial cells. (2000)

   Biochim et Biophysica Acta, vol. 1497(2):261-70.

   (with Chan LN, Wang XF, Chan HC.)

4. Local regulation of anion secretion by pituitary adenylate cyclase-activating polypeptide in human colonic T84 cells. (2001)

   Cell Biology International, vol. 25(2):123-9.

   (with Leung PS, So SC, Lam SY, Chung YW, Chan HC.)

List of Figures

List of Tables

Abbreviations

ABC	ATP-binding cassette
AVP	vasopressin
$CaCl_2$	calcium chloride
cDNA	complementary deoxynucleic acid
CE-LIF	capillary electrophoresis – laser induced florescence
CF	cystic fibrosis
CFTR	cystic fibrosis transmembrance conductance regulator
Cl^-	Chloride ion
dNTP	deoxynucleoside 5'-triphosphate
dsDNA	double strand deoxynucleic acid
DNA	deoxynucleic acid
DPC	diphenylamine-2-carboxylic acid
ENaC	epithelial sodium channel
αENaC	alpha subunit of ENaC
βENaC	beta subunit of ENaC
γENaC	gamma subunit of ENaC
FBS	fetal bovine serum
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
I	current
I_{SC}	short-circuit-current
KCl	potassium chloride

KH_2PO_4	potassium dihydrogen phosphate
K-H solution	Krebs-Henseleit solution
MDR	multi-drug resistance gene
$MgSO_4$	magnesium sulfate
MOPS	3-(N-morpholine)-propanesulfonic acid
Na^+	sodium ion
NaCl	sodium chloride
$NaHCO_3$	sodium bicarbonate
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
mRNA	messenger ribonucleic acid
rRNA	ribosomal ribonucleic acid
RNA	ribonucleic acid
RT	reverse transcription
V	Voltage

Chapter 1: Introduction

1.1 The Human Uterus

The female reproductive system consists of vagina, uterus, oviduct and two ovaries. The uterine wall consists of three layers: endometrium (inner mucosa), myometrium (middle muscular) and perimetrium (outer serosa). The uterus is responsible for implantation, protection, development of the embryo and evacuation of foetus and sperm movement and capacitation. These functions are achieved by the myometrium and endometrium.

1.1.1 Myometrium

The myometrium is the thickest layer of the uterine wall. It is highly vascular and composed of bundles of smooth muscle fibres separated by thin strands of connective tissue. The muscle fibres are arranged to form three interlaced layers: circular, longitudinal and spiral. They promote evacuation of foetus and are important after childbirth as the contracting muscle compresses the blood vessels and prevent haemorrhage.

1.1.2 Endometrium

The endometrium is subdivided into two layers. The upper layer which sloughes off during menstruation is called the functionalis and the lower layer which regenerates the functionalis in the next menstrual cycle is called the basalis.

1.2 The Human Endometrium

1.2.1 The Structure of Human Endometrium

The endometrium is the mucous lining of the uterine cavity. It is composed of two layers: functionalis and basalis (Fig 1.1). The functionalis sheds during menstrual cycle whereas the basalis regenerates after the cycle. The endometrium consists of luminal columnar secretory epithelium overlaying connective tissue and stroma. The stromal cells are spindle in shape. They join to one another in a meshwork of reticular fibres and fine collagenous fibres. Some areas of the epithelium divert and coil into the deep portion of the stroma to form long tubular glands which can only be seen in cross sections (Fig 1.1)

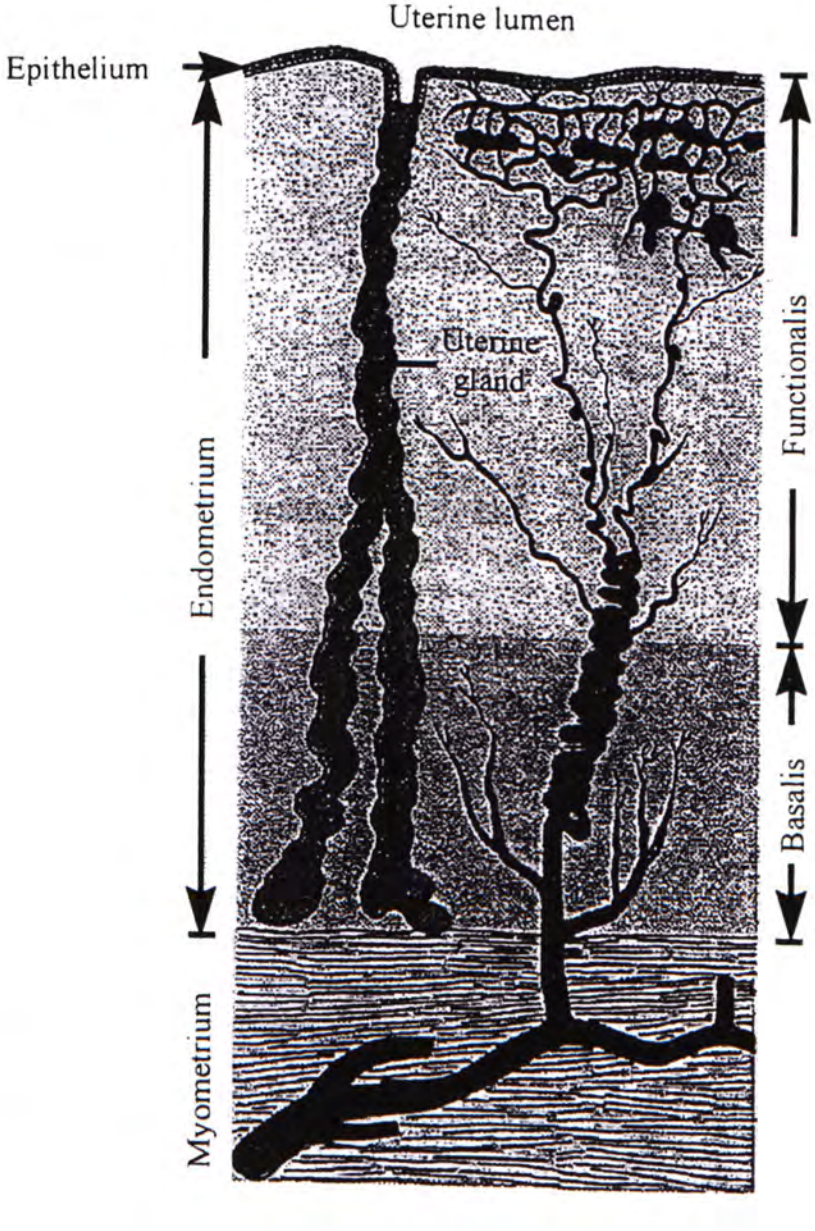


Fig 1.1 Sectional view of the human endometrium.

1.2.2 Cyclic Changes in the Endometrium

Cyclic changes in the secretion of gonadotropic hormones from the anterior pituitary cause the ovarian changes during the monthly cycle. These gonadotropic hormones include the luteal hormone (LH) and follicle stimulating hormone (FSH) (Fig 1.2). The ovarian cycle is accompanied by cyclic changes in the secretion of estradiol and progesterone, which interact with the hypothalamus and pituitary to regulate gonadotropin secretion. The cyclic changes in ovarian hormone secretion cause functional and morphological changes in the endometrium during the menstrual cycle.

The cyclic activity in non-pregnancy can be divided into three phases: (1) the proliferative phase; (2) the secretory phase; and (3) the menstrual phase (Fig 1.2).

In the proliferative phase, increasing amount of estradiol secreted by the developing follicles stimulate the growth of the stratum functionale in the endometrium, where the spiral arteries are also developed. Estradiol may also stimulate the production of receptor proteins for progesterone in preparing for the next phase of the cycle. In the secretory phase, increased progesterone secretion stimulates the development of the mucous glands. As a result of the combined actions of the estradiol and progesterone, the endometrium following ovulation becomes thick, vascular, and "spongy" in appearance. In the menstrual phase, cellular death and sloughing of the stratum functionale in endometrium occurred as the constriction of the spiral arteries, which is responsible for the bleeding during menstruation.

It is believed that the cyclic changes in the endometrium help to prepare the

endometrium for sperm movement as well as nurturing and implantation of the blastocyst. Menstruation also helps to protect the uterus and oviducts from pathogens transported by the sperm (Profet 1993, Strassmann 1996). This is through dislodging the infected endometrial tissue and regenerating endometrial functionalis to deliver immune cells to the uterine cavity. Furthermore, menstrual cycling is an energy saving process, it costs less to renew the endometrial blood and nutrient supply rather than to maintain the endometrium in the metabolically active state for implantation (Strassmann 1996).

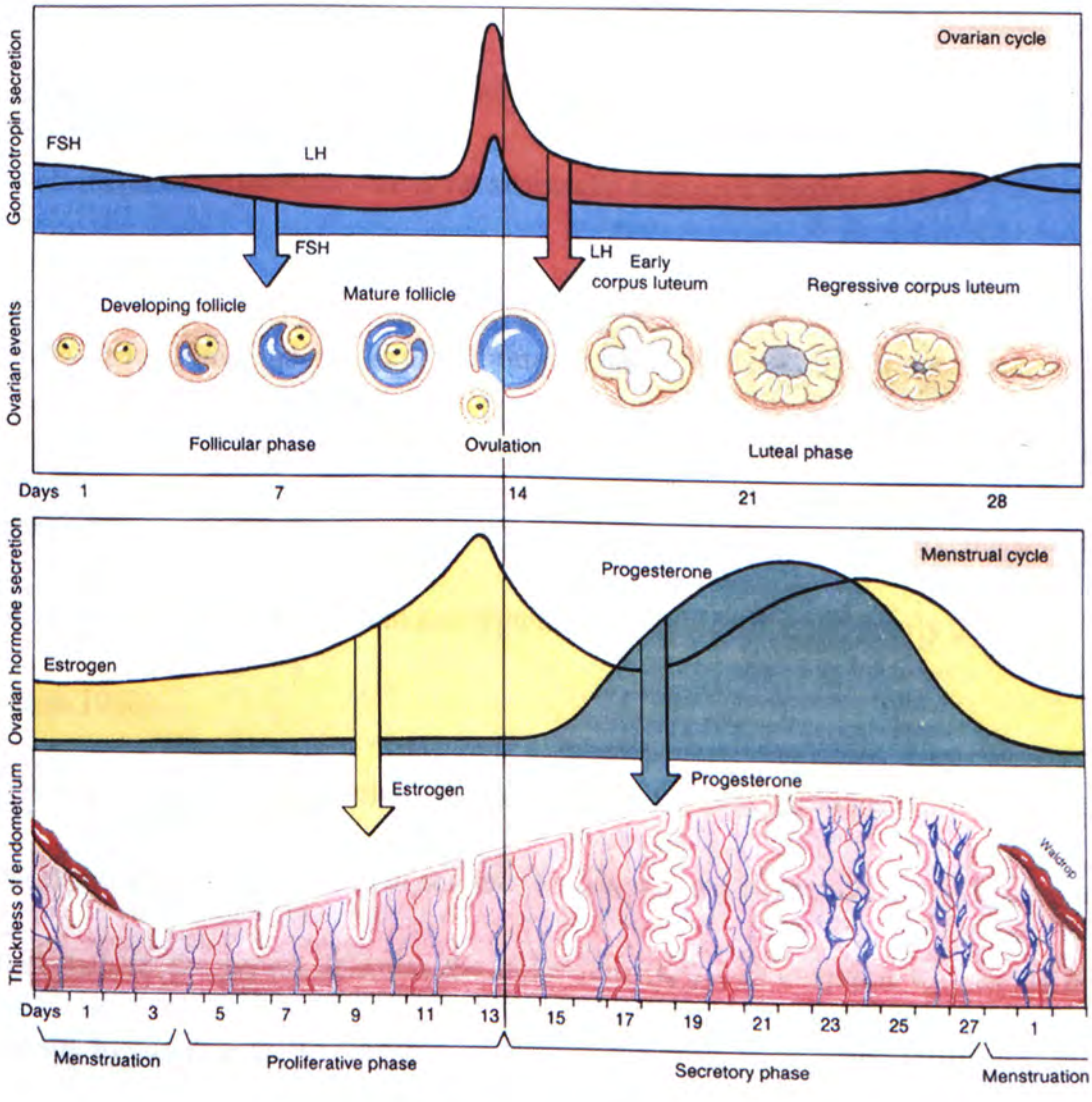


Fig 1.2 Cyclic changes of the endometrium.

1.2.3 Physiological Roles of the Endometrium

The endometrium plays an important role in reproduction. It provides an optimal microfluid environment for sperm movement and capacitation, successful implantation and development of the blastocyst. A precise coordination between the sequence and timing of estrogen and progesterone action on the endometrium provides a well-prepared endometrial cavity for successful blastocyst implantation.

1.2.4 Uterine Fluid Volume and its Composition

Uterine fluid, secreted by the endometrial epithelial cells lining the inner surface of the uterus, provides the optimum environment for sperm movement and blastocyst implantation, but the mechanism and regulation of its secretion are poorly understood (Leese 1988).

The composition of uterine fluid has not been clearly understood. It has been reported that the concentration of sodium (Na^+) was about 30 mmol/L lower in uterine fluid than in the serum, whereas the potassium (K^+) concentration was about 20 mmol/L higher than the serum level. A lower calcium (Ca^{2+}) content and comparable chloride (Cl^-) content compared to that in the plasma have also been recorded (Casslen & Nilsson 1984). Moreover, it has been also found that the human uterine fluid contains several amino acids at high concentrations (Casslen 1987).

1.2.4.1 Regulation of Uterine Fluid Volume and Composition

In secretory epithelia, Cl^- ion movements from the basolateral to apical poles of the cells play a significant role in providing the driving force for fluid movement

(O'Grady et al 1987, Quinton 1990). Water follows the ion movements towards the osmotic equilibrium and thus accumulates in the lumen (Quinton 1990). It has been reported that inhibition of Cl^- flux reduced the rate of fluid secretion (Dickens & Leese 1994).

The uterine fluid undergoes cyclic changes in both volume and composition (Beier 1974). It was found that uterine fluid accumulated in the cavity under estrogen stimulation, disappeared in the luteal phase under the influence of progesterone and re-accumulates pre-menstrually, presumably due to the increased transudation from degenerating endometrial tissue (Casslen 1986). The significance of the reduction of uterine fluid volume and the consequent narrowing of the distance between the epithelial surface in the luteal phase may reduce motility of the embryo in the uterine cavity during the pre-implantation period and to ensure implantation successfully.

In addition, it has been reported that ovarian hormones (17β-estradiol and progesterone) regulate the uterine fluid volume during estrus cycle (Clemetson et al 1977). 17β-estradiol causes secretion of fluid containing sodium, potassium and water into the lumen of the uterus (Meglioli 1976, Shih et al 1940). It has been clearly reported that 17β-estradiol treatment increases the uterine fluid in rats and mice (Casslen 1986). Progesterone has been reported to decrease the volume and increase the viscosity of the uterine luminal fluid; therefore promote reabsorption of uterine luminal fluid (Armstrong 1968, Tantayaporn et al 1974). It has been reported that when progesterone was injected into the rat, or during early pregnancy where progesterone level is comparatively high, the uterine fluid volume is reduced (Casslen

1986).

1.2.4.2 Role of Endometrial Epithelium in the Regulation of Uterine Fluid Volume

Endometrial epithelial cells are responsible for the transfer of nutrients, ions, fluid and diffusible molecules between the circulatory system and uterine lumen, which in turn, optimize, the intra-luminal fluid environment for sperm passage and embryo development and implantation. Although various roles for the uterine fluid have been considered, such as facilitation of sperm transport and participation in sperm capacitation (Kirton & Hafs 1965, Noyes 1953), however, the transport properties of the endometrial epithelial cells, such as the regulation of ion channel expression have not been extensively investigated. Little is known about the mechanisms underlying fluid formation and its regulation, and thus the physiological significance of fluid and electrolyte transport across the endometrium in any species.

1.3 Epithelial Ion Channels

Epithelial ion channels permit the passage of ions and ultimately water across the epithelial barrier. The polarized nature of epithelial cells allows them to play a major role in the regulation of whole-body salt and water homeostasis by separating compartments and maintaining ion gradients (Fuller et al 1999).

Different epithelial cells show different polarity (Cereijido et al 1989a, Cereijido et al 1989b). Plasma membrane is divided into two major domains, the apical and the basolateral. In some instances, it is useful to subdivide the latter into basal and lateral. The endometrial epithelial cells also show these compositional variations reflecting

functional differences. For endometrial epithelial cells, (1) the apical domain is involved in the uptake of nutrients, ions and water secretion towards the uterine lumen, and the interactions with the embryo. (2) The basal domain is responsible for confining the cell to the underlying matrix and receiving signals from the stroma. (3) The lateral domain is responsible for adhering neighboring cells within the epithelium. Compositional differences between apical and basolateral domains are preserved by tight junctions, which prevent the exchange of membrane glycoprotein and glycolipid (Griepp et al 1983, Pinto & Kachar 1982).

The net transport of solute across the epithelia requires a driving force and a transport pathway. There are two types of driving force involved in membrane transport, they are electrochemical potential difference (passive transport) and energy-dependent active transport (active transport). The electrochemical potential difference (passive transport) for an ion is established by the concentration gradient and the difference in electrical potential that exists between the two compartments. The energy-dependent active transport (active transport) is the net movement of a substance that occurs either along or against the existing electrochemical gradient, that is, active transport requires energy input such as ATP hydrolysis. The direction of net transport is determined by the direction of the overall driving force. The system reaches equilibrium when there is no net driving force exerting its effect on the transport pathway.

In the endometrial epithelium, Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), cAMP-regulated Cl^- channel, and Epithelial Sodium Channel

(ENaC) are responsible for secreting and absorbing electrolytes and water, respectively. And we will dicuss this later.

1.3.1 Epithelial Cl^- Channels in Secretory Epithelia

Chloride (Cl^-) channels in epithelial cells can either be absorptive or secretory depending on which membrane they are located, apical or basolateral. Chloride channels are expressed at the apical membranes of the secretory epithelia. A variety of hormones and neurotransmitters, such as prostaglandin E_2, adrenaline and ATP, are known regulators of Cl^- channels (Greger & Kunzelamnn 1990).

Cl^- secretion has been proposed to be coupled to the secondary active transporter Na^+-K^+-$2Cl^-$ co-transporter, which is located at the basolateral membrane (Silva et al 1977). The electrochemical Na^+ gradient generated by the Na^+-K^+-ATPase across the plasma membrane is used to drive the Na^+-K^+-$2Cl^-$ symport. As shown in Fig 1.3, the downhill movement of Na^+ is used to drive the movement of Cl^- into the cell. This accumulates excessive Cl^- in the cytoplasm. When the apical Cl^- channel is open, Cl^- can move down the concentration gradient to the lumen. The excess negative charges accumulated in the apical compartment are neutralized by a flow of Na^+ from blood to lumen through the tight junction. Here the basolateral K^+ channels appear to complete the current loop. The end result is the flow of water from blood to lumen down the osmotic hill.

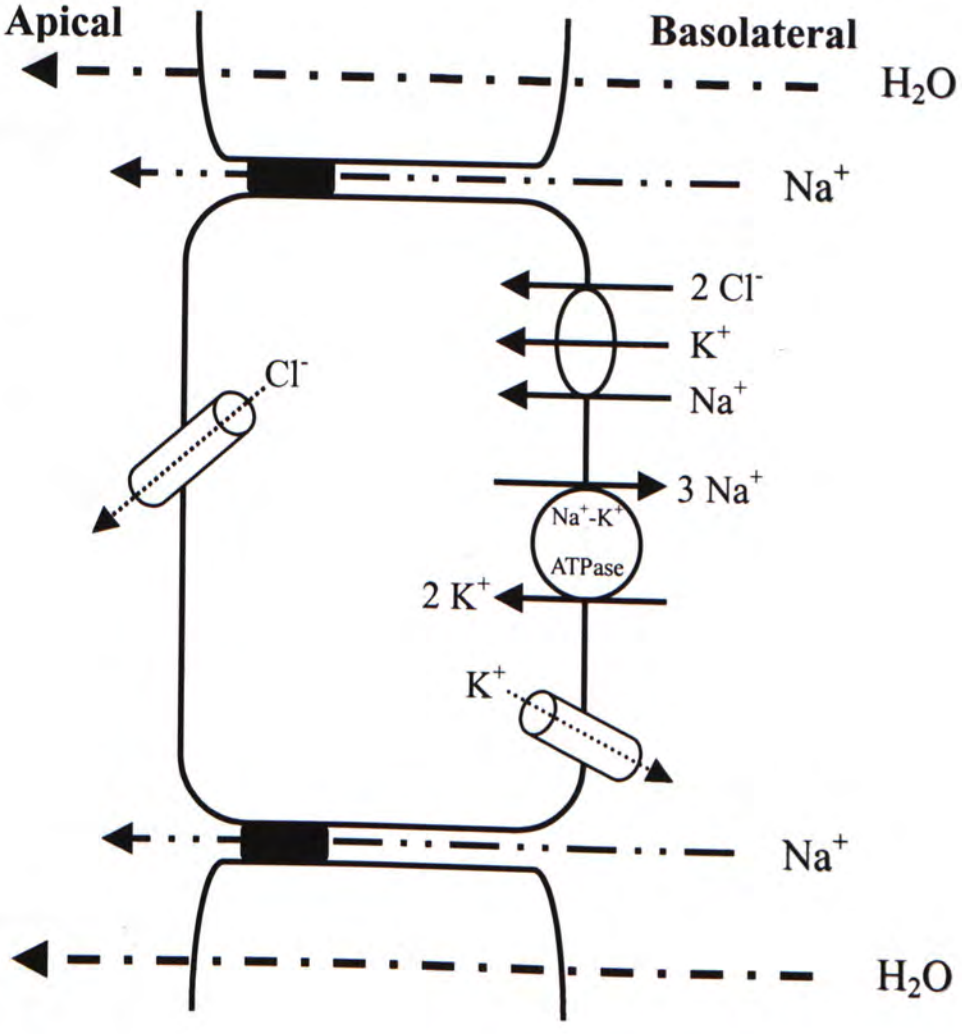


Fig 1.3 Schematic diagram showing epithelial Cl^- secretion.

1.3.1.1 Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

The most well-known cAMP-activated epithelial chloride channels is Cystic Fibrosis Transmembrane Conductance Regulator (CFTR), which is widely expressed in the apical compartment of most epithelia, including pancreatic ducts, small intestinal epithelia, and Cl^- secreting epithelial cell lines (Welsh et al 1992, Crawford et al 1991, Denning et al 1992, Marino et al 1991). Localization of CFTR in the apical membrane allows it to directly mediate Cl^- transport.

CFTR is a membrane protein with two ATP-binding domains and, like the product of the multi-drug resistance gene, MDR, belongs to the ATP-binding cassette (ABC) transporter gene family. Although CFTR has the structure of a transporter that transports substance across the membrane in a nonconductive manner, CFTR also has the intrinsic ability to conduct Cl^- at much higher rates, a function unique to CFTR among this family of ABC transporters (Schwiebert et al 1999).

The most lethal genetic disease in Caucasian associated with impaired electrolyte transport by CFTR is cystic fibrosis (CF), which is identifiable by excessive salt in sweat (Quinton 1990, Welsh & Smith 1995). CF gene is resided on chromosome 7, which normally gives rise to the protein CFTR. CF is the result of a three nucleotides deletion, this alteration, known as the ΔF508 mutation, is the deletion of one amino acid – phenylalanine at position 508 – in the CFTR protein (Fig 1.4). Phenylalanine is lost because the protein-making machinery of the cell now sees ATT at the gene region coding for the protein's 507^{th} amino acid, followed by the GGT sequence for the glycine that normally follows phenylalanine.

The inherited genetic abnormality of CFTR can damage the lungs and cause serious impairment of the pancreas, intestines and liver as well. For example, in the lung, the bronchial tubes and bronchioles become obstructed. Those passages are usually bathed with a thin layer of mucus that traps inhaled particles and carries them to the throat for removal, where in CF patients, the mucus is excessively thick and resistant to remove. This change by itself can narrow air passage and impair breathing. Moreover, when bacteria remain in the air passages, they can induce infections readily. Finally, chronic infection progressively destroys the bronchial passages and, together with the plugging of airways leads to respiratory failure (Fig 1.5).

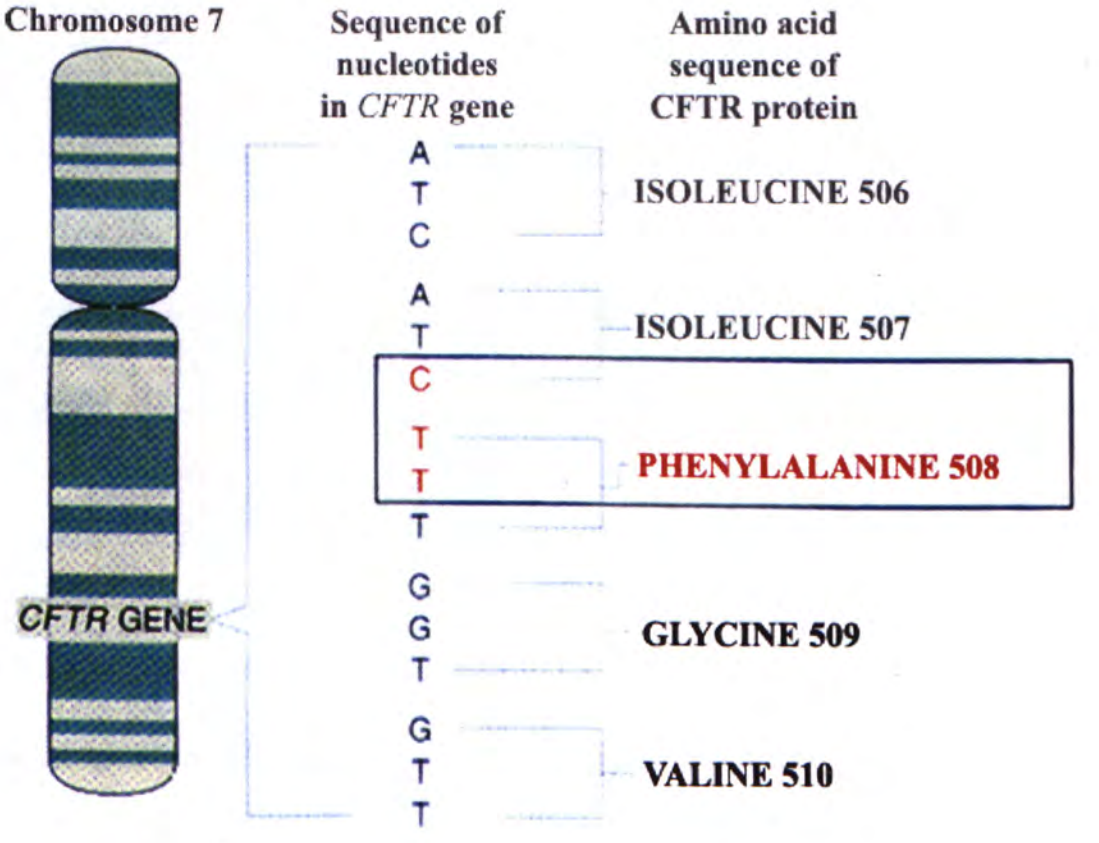
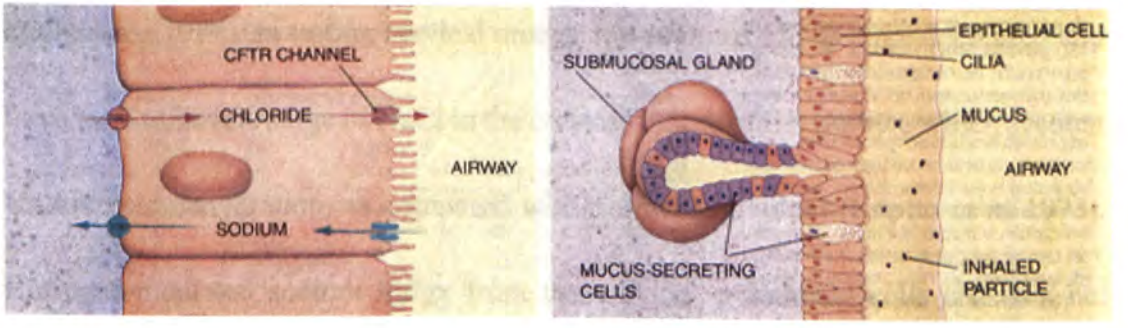


Fig 1.4 Defect in cystic fibrosis gene in chromosome 7. The disease is the deletion of three nucleotides from the gene (*red letters in center column*). (Welsh & Smith 1995)

(a)



(b)

Fig 1.5 Molecular Basis of Lung Disease. The epithelia are shown on left panel and the section of epithelium and air passage on right panel. (a) In healthy individuals, the main epithelial cells lining the airways display at least two types of channels at the surface facing the air passage, CFTR (releases Cl^- into the passage) and the other (takes up Na^+). This enables mucus made by other cells to remain wet, thin and easy to remove from the airways and so the airways remain open. (b) In patients with CF, absence or malfunction of the CFTR prevents Cl^- secretion to the lumen and indirectly causes cells to take up extra Na^+. Then the mucus becomes thicker and more resistant to remove, hence, bacteria are trapped there and flourish. Finally, it leads to destruction of the airway epithelial cells. (Welsh & Smith 1995)

Almost all CF men are infertile, lower pregnancy rate has also been reported in CF women as compared with normal individuals (Susan M.Brugman & Lynn M.Taussing 1984). In testing cervical mucus, the amounts of NaCl are decreased and there is no midcycle surge of NaCl in the cervical mucus of CF women, which account for intrinsic dehydration, as compared with normal individual (Kopito et al 1973). Hormone-mediated sodium influx from the cervical endothelial cells causes a net luminal water movement and consequent dilution of cervical mucus in normal women. However, the cervical mucus in CF women rarely attains great water content at any time in the cycle. Disturbance of this mucus dilution process is probably due to the defective CFTR function, which results in the increased viscosity of the cervical mucus found in CF patients (Kopito et al 1973). Dehydration of cervical mucus presents a formidable barrier to sperm penetration, and is commonly believed to be the cause of reduced fertility seen in CF women.

Uterus is one of the organs expressing CFTR. Pervious studies in mouse endometrial epithelium have demonstrated the convergence of a number of neurohormonal agents, including prostaglandin E_2 (PGE_2), $PGF_{2\alpha}$, adrenaline and ATP, on CFTR (Greger & Kunzelamnn 1990) indicating an important role of CFTR in uterine function. Moreover, endometrium is under constant influence of ovarian hormones (estrogen and progesterone). Previous reports using in situ hybridization and semi-quantitative RT-PCR have shown the up-regulation and down-regulation of CFTR by E_2 in rodents (Trezise et al 1993) and progesterone in human (Mularoni et al 1995), respectively. In addition, cyclic changes in uterine fluid volume and ion

concentrations do not occur in women with CF (Trezise et al 1993). The cyclic expression pattern of CFTR in uterine epithelia is consistent with the role of CFTR in the regulation of uterine fluid volume. Deviation of uterine fluid volume from normal composition has been associated with reduced fertilization seen in women (Harper 1994). However, the mechanism of the regulation of CFTR has not been elucidated.

1.3.2 Epithelial Na^+ Channels (ENaC) in Absorbing Epithelia

The epithelial Na^+ channel, ENaC, plays an important role in Na^+ homeostasis (Garty & Palmer 1997, Benos et al 1995). ENaC has been reported to be present at the apical membrane of many epithelia such as kidney cortical collecting duct and distal colon, airway, sweat and salivary glands. The functional characteristics of ENaC have been studied in isolated renal tubular segments using patch-clamp techniques and can be defined as a small 4 – 6 pS conductance channel in isotonic NaCl with high selectivity for Na^+ and Li^+ over K^+ (>20:1) and slow gating kinetics. In addition, ENaC is highly sensitive to the diuretic amiloride with an apparent inhibitory constant of 10^{-7} M (Schild et al 1997).

Na^+ reabsorption is achieved by Na^+ concentration gradient, which is established by the Na^+-K^+ pump which actively pumps 3 Na^+ out from the cell and 2 K^+ into the cells. This creates an electrochemical gradient for Na^+ reabsorption. The excess K^+ entering the cell is recycled through the basolateral K^+ channel. Consequently, Na^+ concentration is kept low and K^+ concentration high as relative to the extracellular fluid compartment. Under this circumstance, the epithelia establish a transepithelial

potential difference with apical being negative respect to the basolateral membrane. This polarized arrangement of ion channels and transporter allows physiological Na^+ reabsorption which results in a net transport of Na^+ through ENaC from apical to basolateral compartment of the epithelia. As shown in Fig 1.6, this accumulation of Na^+ on the basolateral compartment generates an osmotic gradient for the movement of water from apical to basolateral side of the epithelia.

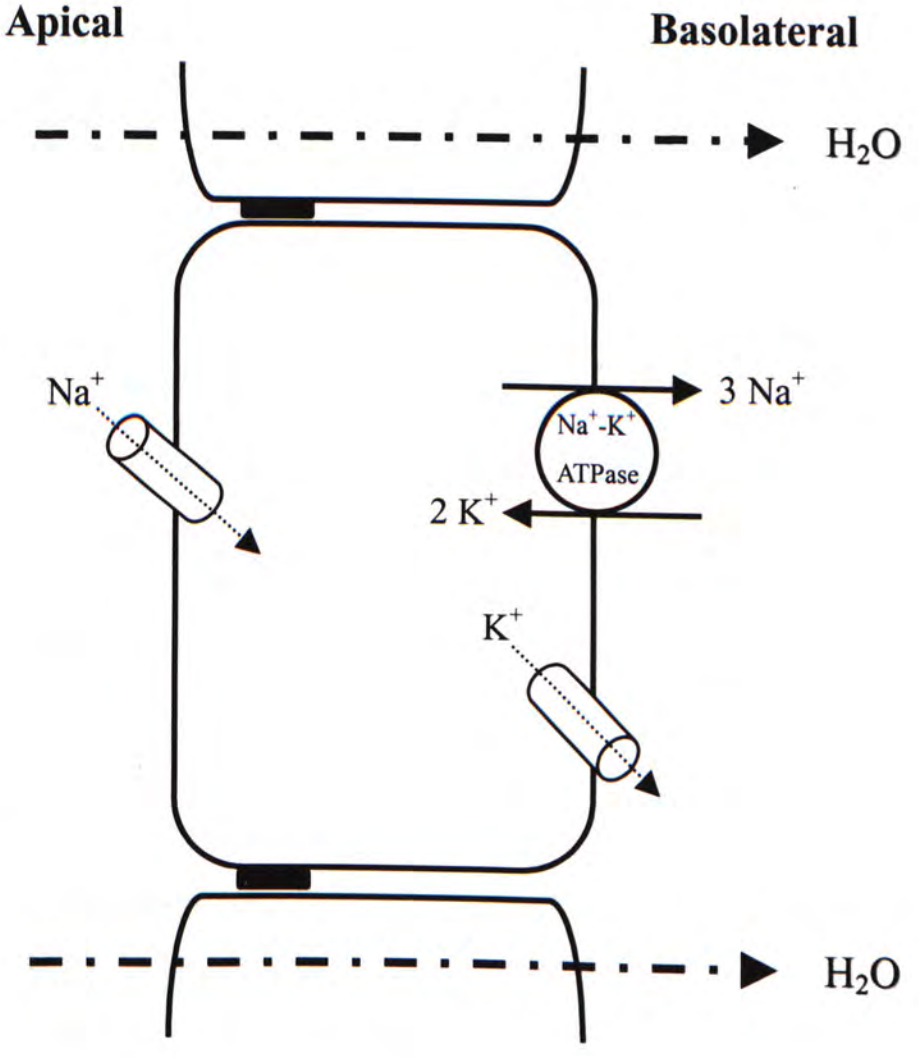


Fig 1.6 Schematic diagram showing Na^+ absorption in epithelia.

The ENaC has different functional roles in various organs in which it is expressed. In the kidney (cortical collecting duct), the modulated reabsoprtion of Na^+ through ENaC provides the primary mechanism of the regulation of urinary Na^+ excretion and thus allows the fine control of the whole organism Na^+ balance under the hormonal control of aldosterone. ENaC has a similar functional role in the distal colon, it prevents excessive Na^+ loss in the stool. In airways, the most important role of ENaC is the reabsorption of the fluid that fills the airways at birth, and thus promotes the shift from fluid secretion (before birth) to fluid reabsorption (postnatal). Working with the CFTR, ENaC also participates in the delicate regulation of the fluid balance in the airways that maintain a thin mucosal fluid film necessary for the clearance of the airway. In the excretory ducts of sweat and salivary glands, the activity of ENaC tends to decrease the luminal Na^+ concentration, prevents major loss of Na^+ in the sweat fluid and allows the excretion of less salty saliva (Hummler & Horisberger 1999).

Recently, the cloning of ENaC from mouse kidney shows that ENaC consists of three homologous subunits called α-, β- and γ-ENaC (Ahn et al 1999). Each subunit has two membrane-spanning regions with the amino terminus and the carboxy terminus inside the cell (Kosari et al 1998). In addition, a large extracellular loop between the membrane-spanning domains contains glycosylation sites and cysteine-rich regions (Fig 1.7). The extracellular regions of these subunits can interact with substances in the extracellular environment and modify the function of ENaC (Stokes 1999). A region of the extracellular loop near the second membrane-spanning domain appears to act as a selectivity filter and might contain a binding site for

amiloride (Schild et al 1997). The three subunits come together to form a channel in ways that are not completely understood. Figure 1.8 shows an oversimplified schematic diagram of how each of the three subunits might interact to form a channel. It is suggested that ENaC has a tetrameric structure and is composed of two α-, β-, and γ-subunits. It has been reported that the three different subunits of ENaC can assemble with different stoichiometries to perform substantially different functions (McNicholas & Canessa 1997). The relative amount of mRNA for each of the subunits can vary widely in various sites, such as lung, kidney, colon and urinary bladder (Watanabe et al 1999, Kopp et al 1998, Watanabe et al 1998), raising the possibility that different stoichiometries can impart different functional consequences. Expression of the α-ENaC subunit alone in *Xenopus* oocytes produced a small amiloride-sensitive current (McDonald et al 1994). In contrast, expression of β- and γ-ENaC, either alone or in combination, did not produce Na^+ current. But, coexpression of γ- with α-ENaC or all three subunits greatly increase Na^+ current in human kidney epithelial Na^+ channel (McDonald et al 1995). It is suggested that the α-subunit is a pore-forming subunit which contains all the biophysical and pharmacological properties of ENaC (Firsov et al 1996). However, the functional role of the β- and γ-subunits has not yet been clearly established. From their structural homology with the α-subunit, it is possible that the β- or γ-subunits also participate in the formation of the channel pore. On the other hand, the ion conduction pathway could be made exclusively of α-subunit with the β- or γ-subunits representing accessory subunits that modulate ENaC activity.

Mutations in ENaC gene are causative for two inherited diseases. Mutations that delete or disrupt a conserved motif in the C-terminus of β- or γ-subunit of ENaC cause Liddle's syndrome, a severe form of hypertension associated with ENaC hyperfunction (Bonny & Hummler 2000, Hansson et al 1995). These mutations increase renal Na^+ absorption, at least in part by increasing the number of Na^+ channels at the apical membrane. Conversely, loss-of-function mutations in α-, β-, and γ-ENaC cause pseudohypoaldosteronism (PHA-1), a neonatal salt-wasting syndrome (Bonny & Hummler 2000). It has been demonstrated that activation of CFTR in the epithelia would inhibit ENaC function (Stutts et al 1995). In cystic fibrosis, loss of CFTR function increases amiloride-sensitive current (Boucher et al 1986), which may contribute to the altered NaCl composition in the airway that contributes to the pathophysiology of cystic fibrosis (Figure 1.5).

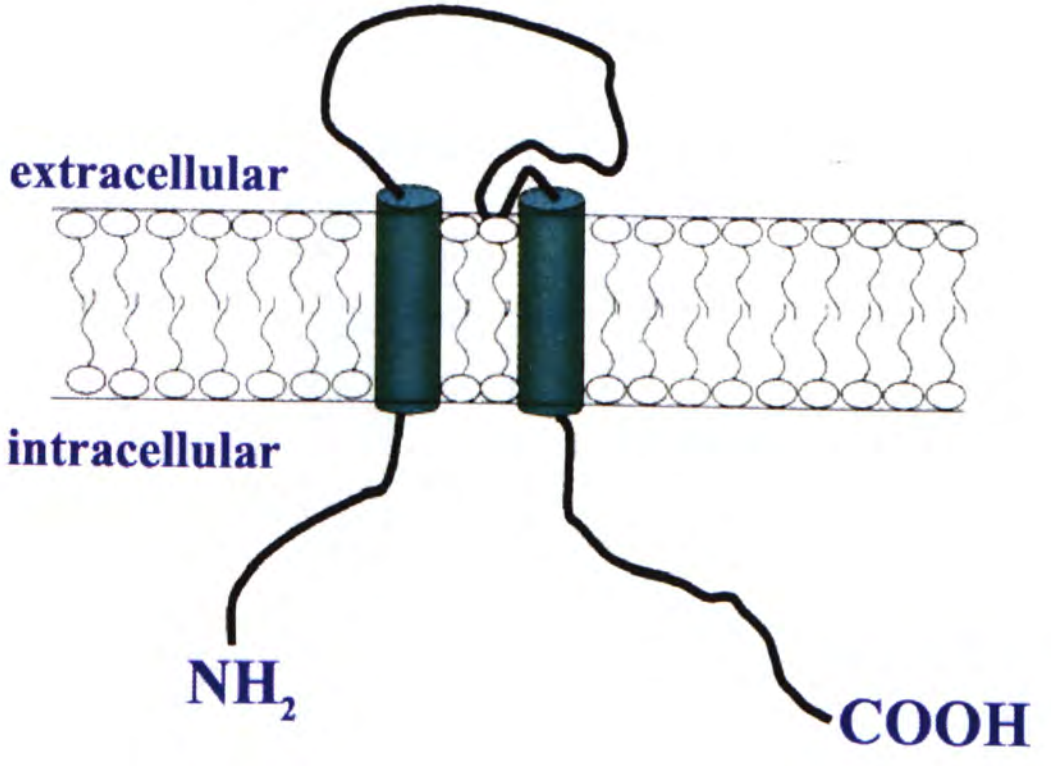


Fig 1.7 ENaC subunit. The general features, two membrane-spanning domains, a large extracellular loop, and intracellular amino- and carboxy-terminal, are typical of all members of this family. (Stokes 1999)

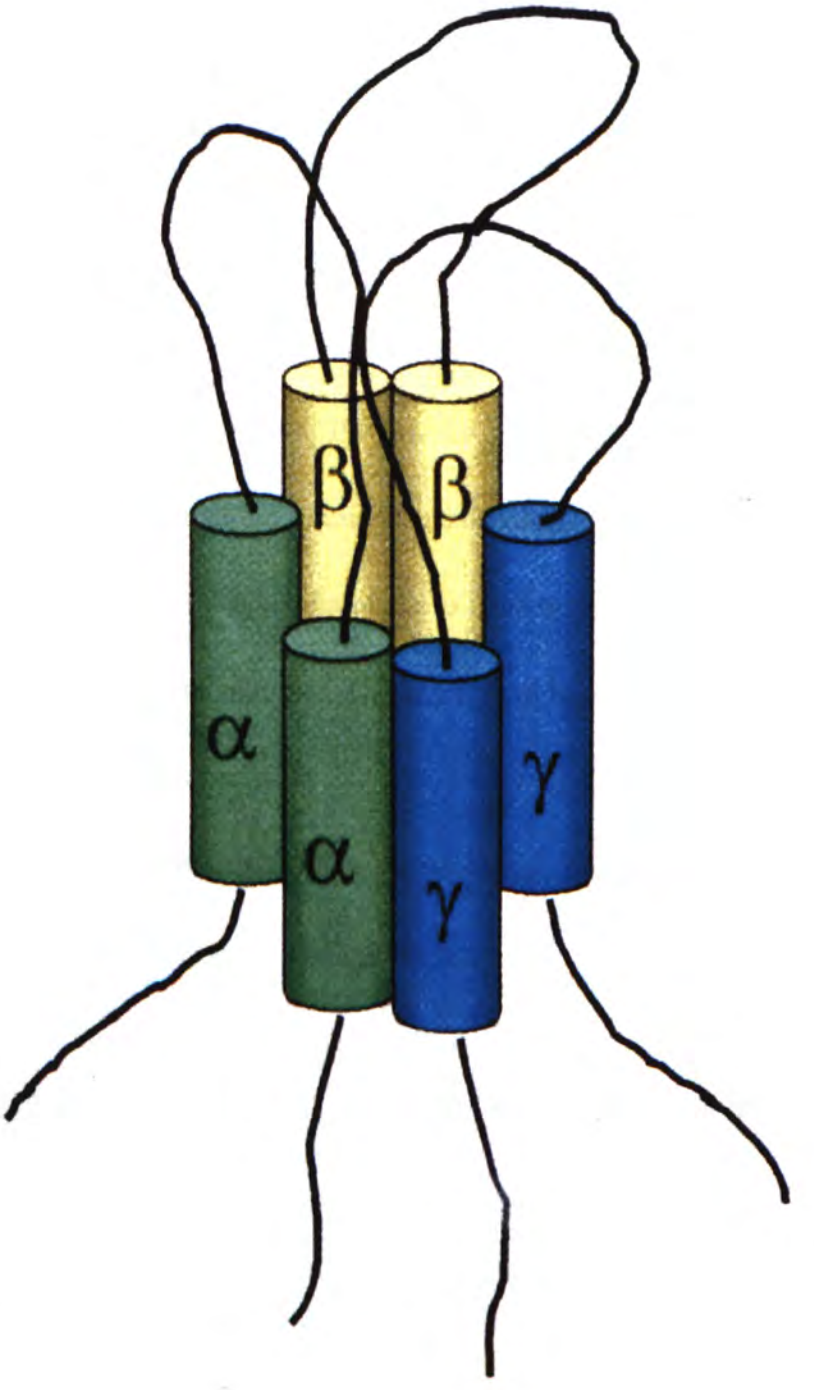


Fig 1.8 Schematic drawing indicating the arrangement of α-, β-, and γ-ENaC into one Na^+ channel. The actual channel complex consists of more than the number of subunits shown here. (Stokes 1999)

1.3.3 ENaC and CFTR in Endometrial Epithelia

The endometrial epithelium is capable of secreting and absorbing electrolytes and water. Previous studies have demonstrated under unstimulated condition, the endometrial epithelia undergo reabsorption of Na^+ from lumen to blood, probably through ENaC (Chan et al 1997b, Matthews et al 1992). Upon hormonal stimulation, the epithelia tend to secrete anions from serosal side to luminal side, probably through CFTR (Chan et al 1997c, Chan et al 1997b). To achieve these dual properties of Na^+ reabsorption and anion secretion under unstimulated and stimulated condition, respectively, the endometrial epithelia ought to have both Na^+ and anion channels on the apical side of plasma membrane. The electrolyte transport model of endometrial epithelial cells can be represented by Fig 1.9.

The co-ordination of these opposing processes determines the luminal fluid environment on which many physiological functions, such as sperm motility and embryo implantation in the uterus, critically depend. The switching between Na^+ absorption and Cl^- secretion is fundamental for epithelial function; however, the mechanism governing these opposing processes is poorly understood. Recently demonstrated inhibition of ENaC by activation of CFTR in a number of epithelia (Mall et al 1996, Ecke et al 1996, Ismailov et al 1996, Kunzelmann et al 1997, Stutts et al 1997, Stutts et al 1995, Letz & Korbmacher 1997) including the mouse endometrium (Chan et al 2000a) has begun to explain how salt and water transport may be regulated by switching from basal Na^+ absorption to Cl^- secretion upon stimulation. The interaction between CFTR and ENaC maybe the underlying mechanisms for balancing

Na^+ absorption and Cl^- secretion across epithelia (Chan et al 2000b, Kunzelmann et al 2000).

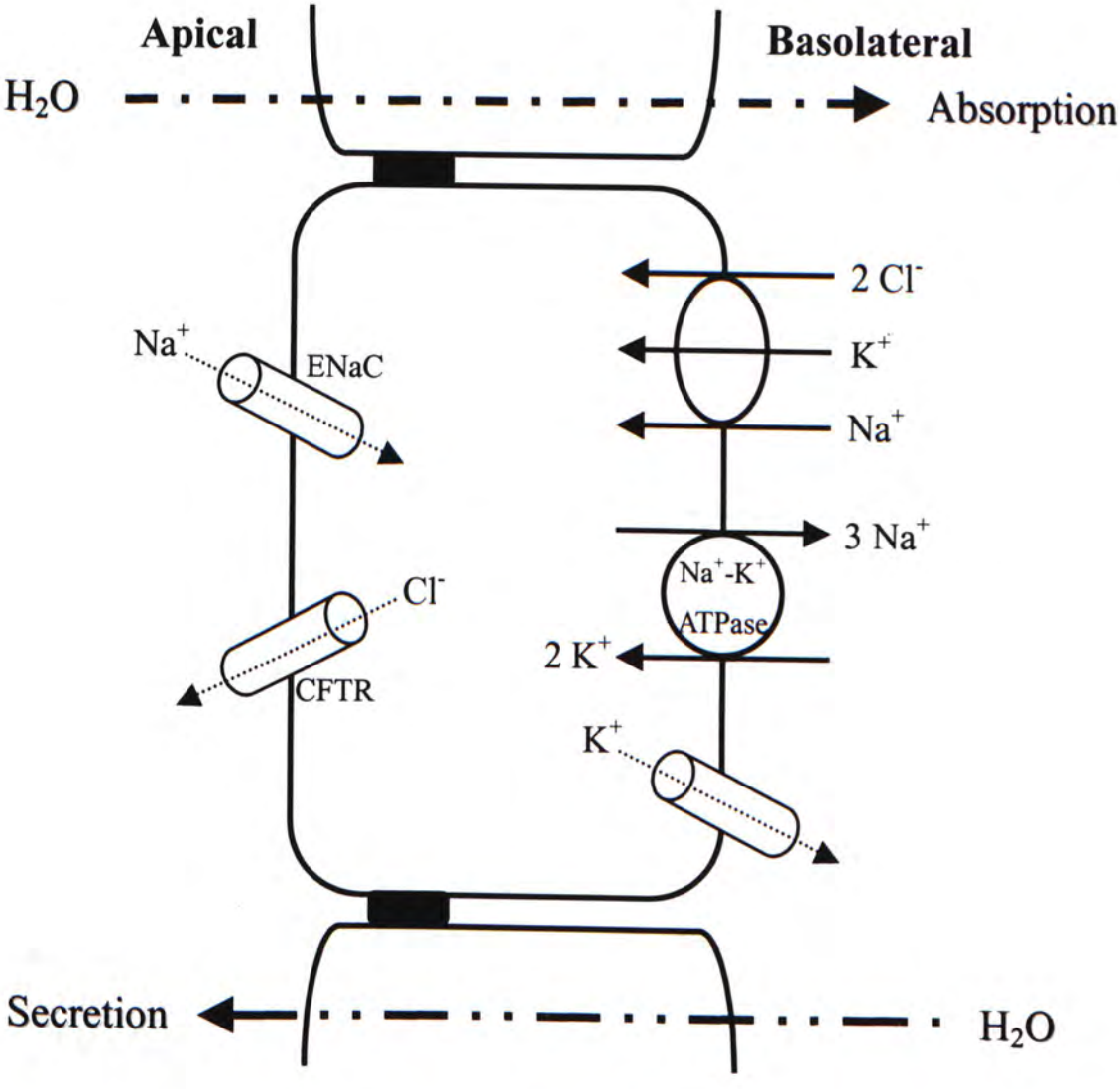


Fig 1.9 Schematic diagram of endometrial ion transport.

1.4 Hormonal Regulation of the Endometrial Epithelium

1.4.1 Estrogen and Progesterone

Estrogen and progesterone are steroid sex hormones, and are synthesized from cholesterol produced by the gonads. Estrogen is responsible for the control of female reproductive processes. The main source of estradiol is the granulosa cells of the mature Graafian follicle, which secretes large quantities of 17β-estradiol. The circulating estrogen bounds loosely to albumin, and it has relatively high metabolic clearance rate. Most of the circulating estrone is derived from estradiol by peripheral 17-hydroxysteroid dehydrogenases.

Progesterone is the steroid hormone primarily responsible for the maintenance of normal pregnancy and, in conjunction with estradiol, for the regulation of pituitary gonadotrophin levels (Woodman 1997). It is produced in the corpus luteum. Progesterone can bind to cortisol-binding globulin, but this is largely prevented by the much higher plasma cortisol concentration. Therefore circulating progesterone bounds loosely to albumin.

The effects of steroid hormones on their target tissues are largely due to the ability of these hormones to induce the production of specific proteins in the target cells. Estrogen and progesterone enter cells freely and bind to receptors of the steroid superfamily (Genuth 1993). The estrogen receptor contains 595 amino acids, and the progesterone receptor, 934 amino acids. Once the steroid-receptor complex is formed, it attaches itself to specific portions of particular genes, promoting transcription of mRNAs from those genes (Fig 1.10). The mRNAs enter the cytoplasm, where their

message is translated into protein by ribosomes. Each steroid-receptor complex can initiate the production of a large number of mRNAs, and each mRNA can result in the synthesis of a large number of copies of the protein. Thus a single hormone molecule might easily result in production of several thousand protein molecules.

In general, estrogen stimulates endometrial proliferation, maturation and desquamation. Whereas progesterone inhibits maturation of the epithelium. The induction of mucification is thought to be the result of estrogen and progesterone working in concert.

The general role of ovarian hormones (estrogen and progesterone) in regulating uterine cycle is well documented. However, their roles in ion channel regulation are sparse. Recent research has shown that CFTR is maximally expressed at proestrus and estrus in rodent uteri where estrogen concentration is high (Trezise et al 1993). Moreover, the role of progesterone in down-regulating CFTR mRNA level has also been studied (Mularoni et al 1995). However, the regulation of endometrial ENaC expression by hormones has not been elucidated. The functional role of CFTR and ENaC and their regulation by the hormones are far from understood.

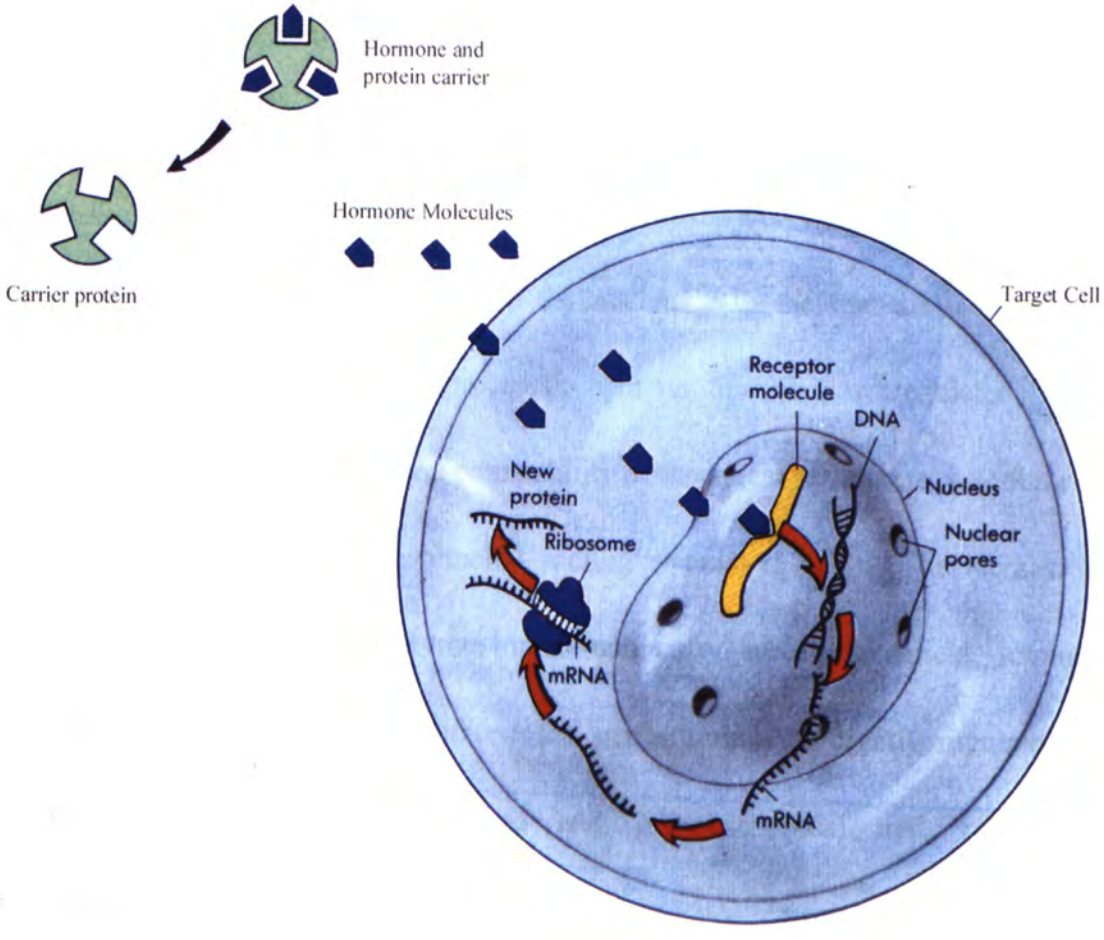


Fig 1.10 The basic mechanism of action of steroid hormones. The hormone penetrates the cell membrane and binds to a nuclear receptor. The hormone-receptor complex enters the nucleus and affects the transcription of DNA coding for specific proteins. (Genuth 1993)

1.4.2 Aldosterone

The mineralcorticoid aldosterone is the primary hormone responsible for maintaining salt and water balance (Funder 1993, Rossier & Palmer 1992). It is produced by the adrenal renal cortex. The major effect of aldosterone is to stimulate Na^+ absorption. Aldosterone induces an increase in transepithelial Na^+ transport by altering gene expression and inducing *de novo* synthesis of particular proteins that mediate its physiological effects (Palmer 1992). As illustrated in Fig 1.11, aldosterone binds to the mineralocorticoid receptor located in the cell cytoplasm, which then becomes activated and translocates to the nucleus. In the nucleus a variety of poorly-understood transcription processes become up- or downregulated; these changes subsequently increase the permeability of the apical membrane to sodium by increasing the activity of the epithelial sodium channel (ENaC). The increase in sodium permeability raises the rate of sodium entry across the apical (luminal) membrane (Stokes 1999). In kidney, Na^+ reabsorption are tightly regulated by corticosteroid hormones (aldosterone and glucocorticoids) and vasopressin (AVP)(Breyer & Ando 1994). Corticosteroid hormones act in their target cells via binding to their receptors (mineralocorticoid receptor, MR, or glucocorticoid receptor, GR); they act as ligand-dependent transcription factors to regulate gene expression (Fig 1.11). This leads to long-term regulation of sodium transport. The molecular understanding of transepithelial sodium reabsorption are (1) ENaC, located in the apical membrane, controls the rate limiting step for transport of salt and water from the tubular lumen, and (2) the Na^+, K^+-ATPase, in the basolateral membrane, which

extrudes Na^+ from the cell to extracellular space. The activity of these transporters depends on aldosterone. In contrast, AVP stimulates rapid sodium (and water) reabsorption, through binding to membrane receptors, production of cAMP, and a cascade of events leading to activation of pre-existing sodium channels (Breyer & Ando 1994)(Fig 1.11). Functional studies have demonstrated the amiloride-sensitive Na^+ current, presumably mediated by ENaC, in both mouse and human endometrial epithelium (Chan et al 1997b, Matthews et al 1998). However, the role of aldosterone in regulating endometrial ENaC has not been investigated.

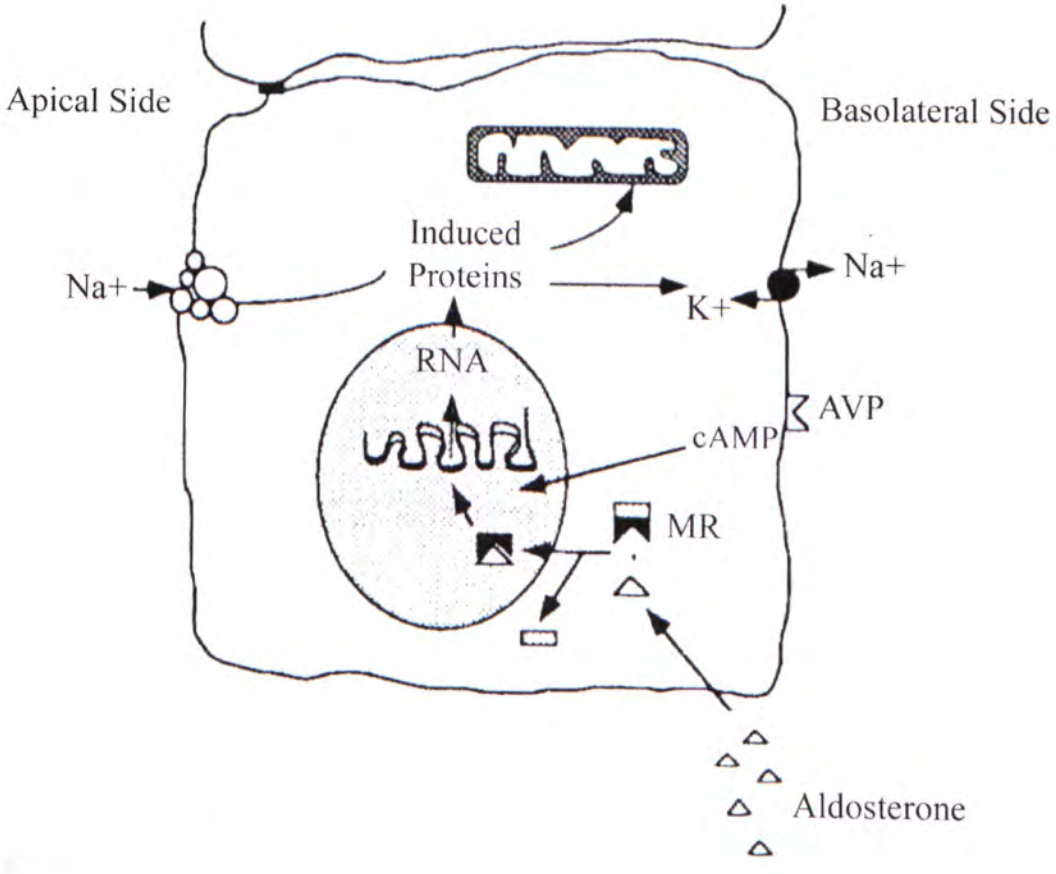


Fig 1.11 Transcriptional regulation pathways by aldosterone and vasopressin (AVP) in a principal cell of the collecting duct. MR, mineralocorticoid receptor. (Breyer & Ando 1994)

1.5 Aim of Study

The endometrium is capable of transferring nutrients, ions, fluid and diffusible molecules between the circulatory system and uterine lumen, which in turns optimizes the intra-luminal microfluid environment for sperm movement and capacitation, embryo development and implantation.

Previous studies in mouse and human endometrium have demonstrated substantial amiloride-sensitive current under unstimulated condition (Chan et al 1997c, Chan et al 1997b, Chan et al 1997a). Moreover, activation of cAMP-dependent Cl^- channel with electrophysiological properties similar to that reported for CFTR has been demonstrated in primary cultured mouse endometrial epithelial cells in whole cell patch-clamp studies (Chan et al 1999). However, the molecular entities and hormonal regulatory mechanism of ion channels in mouse endometrium have not been fully elucidated.

Endometrium is under constant influence of ovarian hormones (estrogen and progesterone). Estrogen is known to play a role in stimulating endometrial proliferation, maturation and desquamation, while progesterone is believed to be involved in inhibiting maturation of the epithelium. However, the roles of ovarian hormones in ion channel regulation are not well studied. It has been reported that CFTR expression could be increased by estrogen (Rochwerger & Buchwald 1993, Trezise et al 1993) and downregulated by progesterone in endometrial epithelial cells (Mularoni et al 1995). However, the roles of ovarian hormones in regulating ENaC expression in mouse endometrial epithelium have not been elucidated. To unveil the

expression pattern of CFTR and ENaC throughout the estrous cycle, the level of mRNA was examined. The effects of ovarian hormones on the expression of CFTR and ENaC were studied both *in vitro* and *in vivo* using short-circuit current technique and semi-quantitative RT-PCR analysis, respectively.

Previous studies have demonstrated that CFTR in epithelia including reproductive tract, could be regulated by various culture conditions, including phenol red in growth media, steroid hormones in serum and Matrigel coated on the filter. It has been reported that phenol red, a pH indicator widely used in growth media, and steroid hormones present in fetal bovine serum, may play a role in cell differentiation and proliferation(Ernst et al 1989, Irwin et al 1991, Kineman et al 1992). Matrigel resembles the extracellular matrix and facilitates rapid epithelial reconstitution and differentiation (Lacy 1995, Rawdon 1998). However, the effects of phenol red and steroid hormones on endometrial ion channels have not been elucidated. One of the aim of the present study was to study the roles of phenol red and steroid hormones in regulating the endometrial epithelial cells by examining the ion channel activities using the short-circuit current technique.

The amiloride-sensitive ENaC, which contribute to the overall Na^+ homeostasis, is located in a variety of epithelia including the kidney, colon, airways, secretory ducts of several glands (Garty & Palmer 1997, Horisberger 1998) and uterus. Aldosterone is the key hormone regulating Na^+ homeostasis in the body. It binds to the receptor that promotes the synthesis of ENaC mRNA in nucleus and then increases the permeability of the apical membrane to sodium by increasing ENaC activity. In order to study a

possible regulatory role of aldosterone in the endometrium, we further examined the expression pattern of uterine CFTR and ENaC mRNA level throughout the estrous cycle of mice fed with a low Na diet.

Mutations in individual ENaC subunits which result in the gain or loss of ENaC function are believed to be the cause of Liddle's syndrome (Hummler & Horisberger 1999) and pseudohypoaldosteronism (Chang et al 1996), respectively. Previous studies have found that the cytosolic termini of the β- and γ-ENaC subunits are involved in the functional interactions between CFTR and ENaC (Ji et al 2000). To further examine the functional role of ENaC subunits, the present study investigated the effect of aldosterone and Matrigel on ENaC activity in addition to that of CFTR by short-circuit current technique, RT-PCR analysis and CE-LIF technique.

It is hoped that the result of the present study may shed light on the understandings of the hormonal regulation of ion channels, and thus electrolyte and fluid transport, in the endometrial epithelium. The results of the studies may provide grounds for the development of contraceptives and possible new treatment for infertility.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Culture Medium and Enzymes

Dulbecco's modified Eagle's medium with nutrient mixture F-12 (D-MEM/F12), phenol red free Dulbecco's modified Eagle's medium with nutrient mixture F-12 (-PR-D-MEM/F12), phosphate buffered saline (PBS), fetal bovine serum (FBS), non-essential amino acid and pancreatin 4xUSP were purchased from GIBCO-BRL (Grand Island, New York, USA) and charcoal/dextran treated fetal bovine serum was purchased from Hyclone. While penicillin/streptomycin and trypsin (type II) were from Sigma Chemistry Co. (St. Louis, MO, USA). Matrigel was acquired from Collaborative Biomedical (Two Oak Park, Bedford, USA).

2.1.2 Drugs

Forskolin and amiloride hydrochloride were purchased from Research Biochemical International (RBI, Nitick, MA, USA). Dipheylamine-2-carboxylic acid (DPC) was purchased from Riedel-de Haen Chemicals (Hannover, Germany). 17β–Estradiol, progesterone and aldosterone were purchased from Sigma Chemistry Co. (St. Louis, MO, USA). Glucose, calcium chloride, magnesium chloride, potassium chloride, potassium dihydrogen phosphate and sodium chloride were purchased from Merck (Darmstadt, Germany).

2.1.3 Molecular Biology

Isopropanol, choloform and etheium bromine were purchased from Merck (Darmstadt, Germany). Diethyl Pyrocarbonate (DEPC) was purchased from Acros Organics (New Jersey, USA). RT-PCR kit, Trizol Reagent Kit, Superscript preamplification system, 100bp DNA ladder, 10x BlueJuice gel loading buffer, Lipofectin Reagent and CFTR, ENaC primers and their antisense primers were purchased from GIBCO-BRL (Grand Island, New York, USA). Taq DNA Polymerase (Thermus aquaticus) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). GeneClean® II kit was purchased from Quantum (BioTechnologies, Inc., CA).

2.1.4 Experimental Tissues and Animals

Uterus horns were removed from 3.5 to 4 week-old female ICR mice which were supplied by the Laboratory Animal Services Centre of the Chinese University of Hong Kong.

2.2 Preparations

2.2.1 Pervious Support for Cell Growth

Endometrial epithelial cells seeded on 0.45 μm nitrocellulose Millipore filters (Type HA025, Nihon Millipore Kogyo K.K., Japan) were used for short-circuit current (I_{SC}) measurement. Functional monolayers were formed by the epithelial cells with apical-basolateral polarity retained. This allowed us to measure transepithelial ion

fluxes by short-circuiting the epithelia.

Silicone rings were used to confine the area for cell growth. They were prepared from Sylgard 184 elastomer kit (Dow Corning Corp., USA). Sylgard resin was mixed well with the curing agent in a 10:1 (w/w) proportion. Each 100 mm petri dish was filled with 9 g well-mixed Sylgard and allowed to set on a horizontal surface for 3 days.

Silicone rings with internal diameter of 7 mm were cut out from the well-set Sylgard plate using a double-barrel circular puncher. Each silicone ring was glued onto a piece of 0.45 μm nitrocellulose Millipore filter using Silicone 3140RTV non-corrosive adhesive (Dow Corning Corp., USA). Each Millipore filter was coated with 100 μl matrigel which had been diluted 8 times by PBS. The assemblies were sterilized under ultraviolet radiation for 30 minutes before seeding cells on them. The sterilized assemblies were allowed to float on a 100 mm petri dish which contained 12 ml completed cultrue medium (Fig 2.1).

2.2.2 Growth Medium

Filter unit with a 0.22 μm Millipore filter layered inside was put on the top of the bottle. The assembled unit was autoclaved at 115°C at 1 atm for 30 minutes and then was dried in the oven.

Dulbecco's modified Eagle's medium with nutrient mixture F-12 (D-MEM/F12) in powder form was kept stirring in a 1 L beaker with 800 ml Nano-pure water inside. Then 1.2 g $NaHCO_3$ was added into the solution. The pH value was adjusted to 7.2, a little bit lower than the desired value 7.4 because there will be a 0.2 – 0.3 rise in pH value after filtering. The solution was made up to 1 L. The medium was filtered into two 500 ml bottles through the sterilized filter unit. Each 500 ml growth medium was supplemented with 100 μM non-essential amino acid, 100 IU/ml penicillin and 100 μg/ml streptomycin, and 10% fetal bovine serum (FBS). The completed growth medium was kept refrigerated.

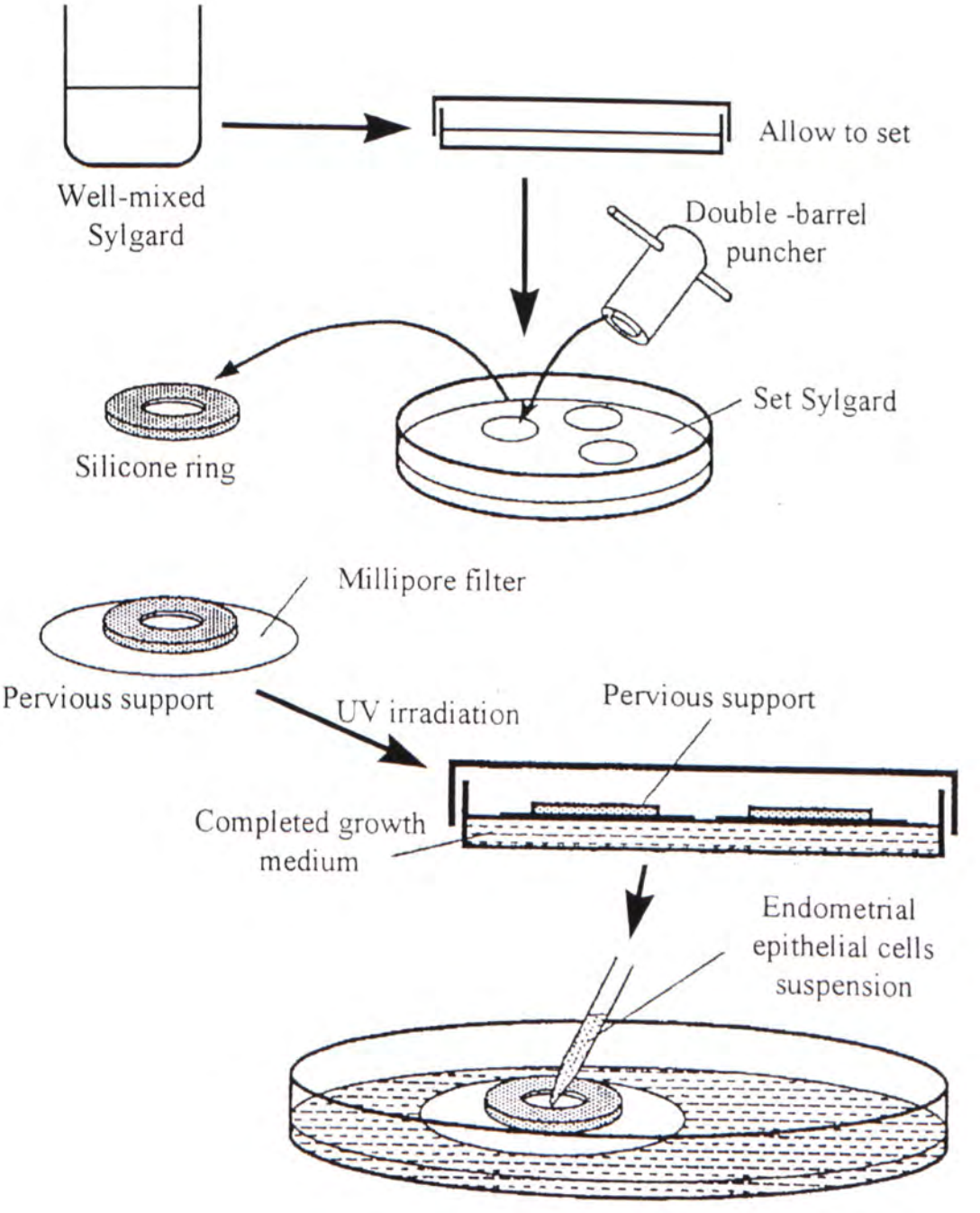


Fig 2.1 Preparation of pervious support for cell growth.

2.2.3 Culture of Mouse Endometrial Epithelial Cells

Endometrial epithelial cells were enzymatically isolated from the mouse uterus according to the method described by McCormack & Glasser (McCormack & Glasser 1980) with slight modifications (Chan et al 1997b). Uteri were obtained from 3.5 to 4 week old immature ICR mice to avoid the complication of the estrous cycle. Uteri obtained were washed in sterile PBS (without Ca^{2+} and Mg^{2+}). After trimming off the fatty and connective tissues, the uteri were sliced longitudinally. The sliced uteri were transferred to a 15 ml centrifuge tube containing 10 ml PBS (without Ca^{2+} and Mg^{2+}) supplemented with 7.5 mg/ml trypsin and 25 mg/ml pancreatin. Tissues were incubation at 0°C for 60 minutes and then at room temperature for another 45 minutes.

The enzyme-containing PBS was poured away carefully, the completed growth medium was added to stop the activity of trypsin. The medium was replaced with PBS 5 minutes later. The tissues were then gently shaken for 30 seconds. Uterine tissues were removed and the suspension was centrifuged at 1000 x *g* for 3 minutes. The supernatant was discarded and the cell pellet was resuspended in 12 ml PBS. The cells were allowed to settle for 5 minutes and the top 2 ml which was rich in red blood cells was discarded. The suspension was centrifuged at 1000 x *g* for another 3 minutes. Then the cell pellet was resuspended in the completed growth medium. The isolated endometrial cells were seeded on a 0.45 cm^2 matrigel-coated nitrocellulose Millipore filters for short-circuit current measurement. Cells were incubated at 37°C in 5% CO_2 / air and studied on the third day when confluence of cell growth was reached.

2.2.4 Solutions for Short-circuit Current Measurements

The Krebs-Henseleit (K-H) solution was used as bathing solution in short-circuit current measurement. The composition and concentration were sodium chloride (NaCl), 117mM; potassium chloride (KCl), 4.7mM; calcium chloride ($CaCl_2$), 2.5mM; magnesium chloride ($MgCl_2$), 1.2mM; sodium bicarbonate ($NaHCO_3$), 24.8mM; potassium dihydrogen phosphate (KH_2PO_4), 1.2mM; glucose, 11.1mM. The pH value of the solutions was kept at 7.4 when gassing with 5% CO_2 / 95% O_2.

2.2.5 Electrodes for the Short-circuit Current Measurement

Current and voltage probes were in fact Ag/AgCl electrodes (World Precision Instruments, Inc., USA) which were inserted into the 3 M KCl-agar salt bridges. In brief, cartridges (World Precision Instruments, Inc., USA) were filled up with 3% agarose (w/v) which was dissolved in 3 M boiling KCl solution. Then the Ag/AgCl electrodes were immediately inserted into the cartridges. No bubbles around Ag/AgCl connections were allowed because the dead space of bubbles generated large resistance, which attenuated both the applied and measured signals. Solidification of agar was achieved by immersing the agar-filled cartridges into 3 M cooled KCl solution. The well-prepared electrodes were short-circuited in 3 M KCl solution so as to minimize the potential difference between two electrodes.

2.2.6 Solutions for the Molecular Biology Experiments

2.2.6.1 Diethyl Pyrocarbonate (DEPC)-treated water

For 1 L nanopure distilled water, 0.1 % of DEPC was added. The solution was stirred with a magnetic stirrer at high speed for at least 5 hours. The solution was then autoclaved and stored at room temperature until use.

2.2.6.2 1x TAE buffer (DNA gel electrophoresis and its running buffer)

For 1 L of 1x TAE buffer, 4.84g Tris base, 1.142g glacial acetic acid and 2 ml 0.5M disodium ethylenediamine tetraacetate dihydrate (EDTA) were mixed with 990 ml deionized water. The solution is adjusted to pH 8.0 then it was made up to 1 L with deionized water.

2.2.6.3 5x MOPS (RNA gel electrophoresis and its running buffer)

MOPS (100 mM), sodium acetate (25 mM) and EDTA (2.5 mM acid free) were dissolved and well mixed with 800 ml DEPC-treated water, the pH of 7.0 was adjusted using NaOH (5M). The volume was adjusted to 1 L with DEPC-treated water. The solution was filtered before use.

2.2.6.4 Formaldehyde gel-loading buffer

50% glycerol, EDTA (1 mM, pH 8.0). 0.25% bromophenol blue and 0.25% xylene cyanol FF were dissolved and well mixed with 10 ml DEPC-treated water.

2.3 Protocol

2.3.1 Effects of Ovarian Hormones and Aldosterone on CFTR and ENaC Expression

In order to study the effect of ovarian hormones (estrogen and progesterone) and aldosterone on uterine CFTR and ENaC, the short circuit current measurement and semi-quantitative RT-PCR were conducted. 17β-estradiol (2μg/100g body wt in saline with 10% ethanol was injected intraperitoneally), progesterone (1.5mg/100g body wt in saline with 10% ethanol was injected intraperitoneally) and aldosterone (2μg/100g body wt in saline with 10% ethanol was injected intraperitoneally) were added to the culture medium (apical and basolater side) or administrated intraperitoneally to the ovariectomized ICR mice 24 hours prior to the start of the experiment.

Ovariectomy are following anaesthesia with ketamine (75 mg/kg i.p.) and xylasine (10 mg/kg i.p.), the anaesthetised animal was laid on its ventral surface and a small midline dorsal skin incision made approximately half way between the middle of the back and the base of the tail. The peritoneal cavity was entered by laying the animal onto its side whilst simultaneously moving the skin incision above the muscle to the side of the body. A small incision was then made with small iris scissors and the ovaries and connected uterine horn removed slightly out of the peritoneum using blunt forceps. The blood supply to the ovaries was then occluded using haemostats clamped onto the oviduct. The ovary was then carefully removed ensuring no ovarian tissue remained and the uterus replaced back into the peritoneal cavity. The procedure was

then repeated for the opposite side and the skin incision closed with Michel clips. Postoperative pain relief was provided with temgesic (0.05 mg/kg s.c.) and the animal left to recover for 3 weeks prior to further experimentation

2.3.2 Possible Interaction between CFTR and ENaC upon Hormonal Stimulation

Short-circuit current measurements were conducted to investigate possible interaction between CFTR and ENaC upon hormonal stimulation. The ENaC activity was revealed by the magnitude of amiloride-sensitive Na^+ current. In the presence of amiloride where contribution of Na^+ was eliminated, the CFTR activity can be reflected by the forskolin-induced current.

2.4 Methods of Measurement

2.4.1 The Short-circuit Current Technique

The short-circuit current (I_{SC}) technique was used to study active transepithelial ion transport across the primary cultured endometrial epithelial cells grown on permeable support. This technique was first introduced by Ussing and Zerahn (Ussing & Zerahn 1951) in studying the active Na^+ absorption of frog skin. Afterwards, this technique was extensively used for studying transepithelial transport in various tissues (Schultz & Zalusky 1964, Clarkson & Toole 1964, Cooperstein & Hogben 1959). This technique allowed us to study the active transepithelial transport of ions in both resting and stimulated conditions.

The main shortcoming of the short-circuit current technique is the inability in resolving individual active transport process that takes place at the same time. Nevertheless, with more and more commercially available specific channel blockers and inhibitors, we are able to study and identify independently the contribution of each individual transporter to the resultant I_{SC} contribution.

2.4.1.1 The Short-circuit Current Setup

The measurement of I_{SC} has been described previously (Ussing & Zerahn 1951, Wong 1988). Primary cultured endometrial cells growing on permeable supports were vertically clamped between 2 halves of the Ussing chamber with a 0.45 cm^2 windows. The assemble was held in position by penetrating stainless steel pins in one half-chamber to the holes in the other half-chamber. Since these pins entered the

silicone ring such that the epithelial cells were not directly pressed again the chamber, edge damage to the tissue was prevented (Fig. 2.2)

Two pairs of outlets (V and I) were connected to the Ag/AgCl electrodes assembled agar bridges for the measurement of transepithelial potential difference and the injection of external current, respectively. The voltage electrodes which measure the open circuit potential difference across the monolayer were positioned in close proximity to minimize the attribution of fluid resistance. Conversely, the current electrodes were situated as far from the epithelium as possible to make sure an uniform current was injected across the epithelium. The two halves of the chambers were held in horizontal position by screws at the end of both sides. (Fig. 2.3)

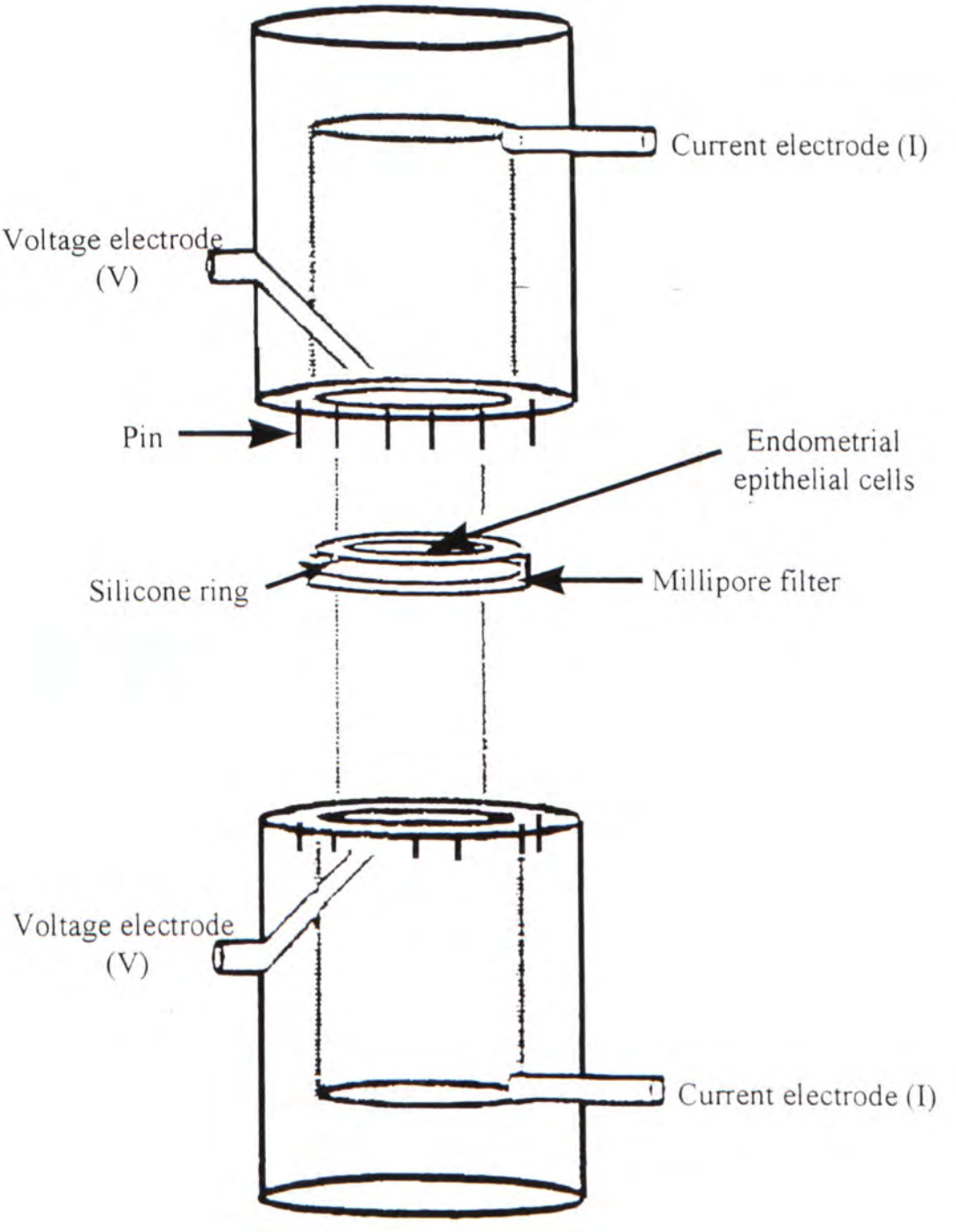


Fig 2.2 Schematic diagram of the Ussing Chamber.

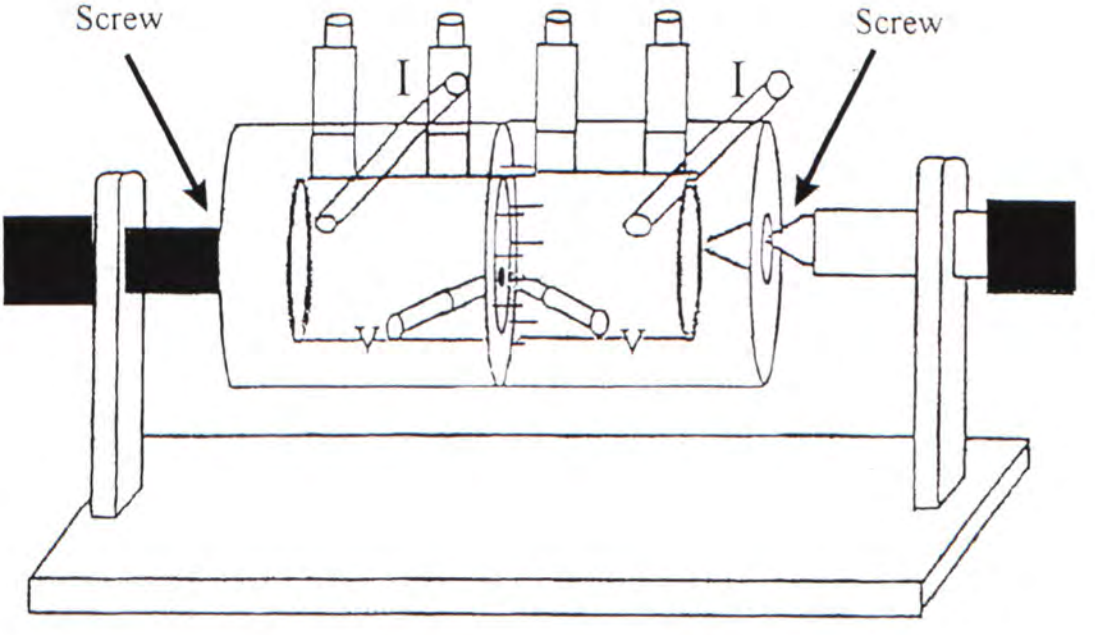


Fig 2.3 The Ussing Chamber in horizontal position. The screws at both ends of the chamber are used to fix the chamber in position.

2.4.1.2 Experimental Procedures

All electrodes were connected to a preamplifer which was connected in turn to a dual-voltage-clamp amplifier (DVC 1000; World Precision Instruments). Voltage offset of two voltage electrodes and fluid resistance were adjusted and compensated prior to the onset of each measurement. Monolayers were bathed on each side with 10 ml Kreb's solution which was maintained at 37 °C by a water jacket enclosed the reservoir. The solution was continuously bubbled with 95% O_2/ 5% CO_2 to maintain the pH of the solution at 7.4 and the circulation in the chamber. A counter current, which is termed short-circuit current (I_{SC}), was applied to the epithelia so as to maintain a 0 mV potential difference across the epithelia (Fig. 2.4). This short-circuit current (I_{SC}) was displayed in real time on chart recorder (BD111, Kipp and Zonen, World Precision Instrument, USA). The transepithelial resistance (R_T) was monitored by clamping the epithelia alternatively at zero and non zero potential. The transepithelial resistance was assumed to obey Ohm's Law:

$$\text{Transepithelial resistance } R_T = \frac{\text{Change in clamping potential } (\Delta V)}{\text{Change in current } (\Delta I)}$$

All epithelial cells are characterized by their compositional variations in plasma membrane among apical and basolateral domains. The differential localization of ion channels, receptors and transporters reflects functional differences. Agonists, antagonists and channel blockers could be added directly to the apical or basolateral

side of the epithelium. The involvement of receptors, ion channels or transporters could be studied and inferred.

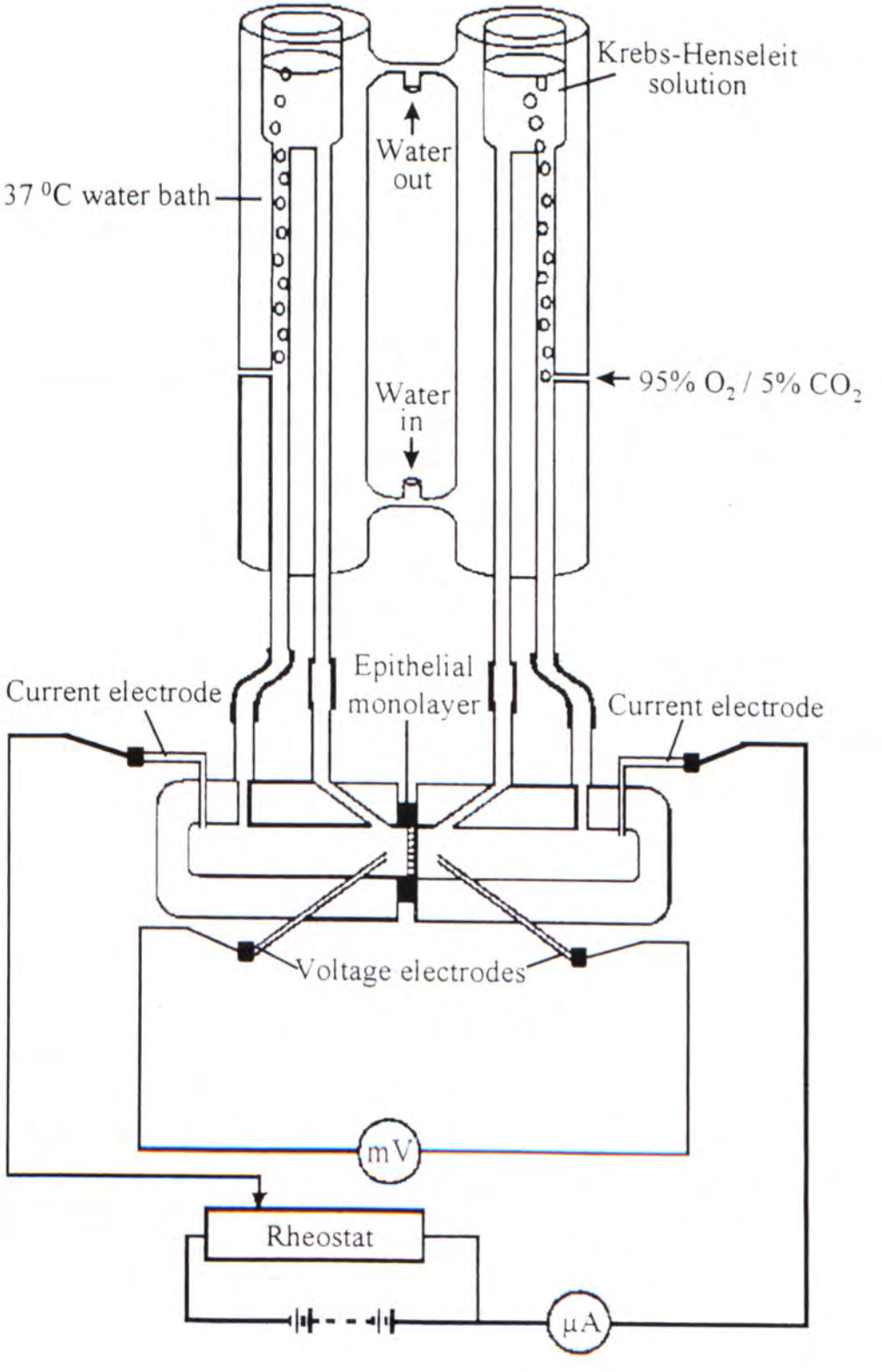


Fig 2.4 Experimental set-up of the short-circuit current (I_{SC}) measurement.

2.4.1.3 Data Analysis

The change in I_{SC} was defined as the maximal rise in I_{SC} following agonist stimulation and it was normalized as current change per unit area of epithelial monolayer by dividing the I_{SC} measured by 0.45 cm^2 (in $\mu A/cm^2$).

2.4.2 Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

2.4.2.1 RNA Isolation

Primary cultured endometrial monolayers and uterus were removed from ICR mice were undergoing RNA extraction.

Endometrial monolayers were scraped off the permeable filters bathed in FBS supplemented growth medium after 4 days culture. Afterward, the cells were spun down at 1000 x *g* for 3 minutes and the supernatant was discarded. On other hands, the uterus was removed from ICR mice and homogenized by Trizol Reagent Kit (GIBCO-BRL, Grand Island, New York, USA). The samples were lysed with 1 ml Trizol Reagent at room temperature for 5 minutes to allow complete dissociation of cell complexes. Then, 0.2 ml of chloroform per 1 ml of Trizol Reagent was added into the sample. The sampling tube was shaken vigorously by hand for 15 seconds. The mixture looked milky pink after shaking. The sample was then centrifuged at less than 14,000 x *g* for 10 min at 4°C. After centrifugation, the sample was distinguished into three heterogeneous layers with milky pink protein at the bottom, white precipitate DNA in the middle layer and clear aqueous phase RNA sample at the top.

The upper RNA phase was carefully transferred into a 1.5 ml fresh DEPC-water treated microcentrifuge tube. Then, 0.5 ml of isopropanol per 1 ml of Trizol Reagent was used to precipitate the RNA after 10 minutes incubation at 4°C and centrifugated at less than 14,000 x *g* for 10 min at 4°C. After centrifugation, the supernatant was carefully removed, and the RNA pellet was washed with 1 ml of 75% ethanol per 1 ml of Trizol Reagent. The RNA pellet was air-dried for 3 - 5 minutes. Finally, the RNA sample was dissolved in Rnase-free water. UV absorbance, which was reflected by optical density (OD), of RNA sample was measured by using UV-spectrophotometer (UV-1201, Shimadzu Corporation, Japan). The concentration of RNA (μg/μl) was calculated as followed:

$$[\text{RNA}] = \frac{\text{Optical depth (OD)} \times \text{Dilution factor} \times 40 \text{ (constant)}}{1000}$$

Where 40 is the constant for RNA analysis at wavelength 260 nm. Agarose gel electrophoresis was performed to see the integrity of the RNA sample. RNA was stored at –70°C for not more than a month for RT-PCR analysis. The process of RNA isolation is illustrated in Appendix A.

2.4.2.2 RNA Gel Electrophoresis

RNA integrity could be checked by ethidium bromide-stained agarose gel electrophoresis. Usually two clear bands corresponding to 18S rRNA (1.87 kbp) and

28S (4.42 kbp) should be seen under UV radiation provided that the RNA sample was not degraded.

To prepare 1.0 % RNA gel, 0.5 g of agarose was added into 40 ml DEPC-treated water. After boiling in a microwave appliance at high power, agarose was dissolved. Then 10 ml 5x MOPS and 15 μl of 1 mg/ml ethidium bromide were added. The gel was allowed to set in a 8-well gel tray (7 cm x 10 cm). RNA sample was prepared by mixing 20 μg RNA sample with 2.6 μl of 37% formaldehyde, 7.5 μl of deionized formamide and 1.5 μl 5x MOPS. The mixture was incubated at 65 °C for 15 minutes and in ice for another 5 minutes. Finally, 2 μl of RNase free loading buffer was added. RNA gel was put in the gel caster (BIO-RAD, Mini-Sub Cell GT System, mini-gel caster, USA) containing cold 0.5x MOPS as running buffer. The treated RNA sample was carefully added into the well of the RNA gel. The gel tank was connected to the power supply (PowerPac 200, BIO-RAD, USA). RNA nucleotide fragments which carried negative charges would migrate to the anode under the electric field. The gel was collected when the dye front has migrated to 2/3 of the gel. RNA fragments were viewed under UV light box. Any blur of the two bands indicated the degradation of the RNA sample which would be discarded.

2.4.2.3 Reverse Transcription (RT)

The purpose of reverse transcription was to transcribe messenger RNA (mRNA) to first strand complementary DNA (cDNA). However, the RNA sample

isolated by the above procedure was composed of total RNAs, including mRNA, rRNA and cRNA. The mRNA with a long poly(A)$^+$ tail could be fished out from the total RNA sample by Oligo (dT) primer. Reverse Transcription was achieved with the Superscript preamplification system (GIBCO-BRL, Grand Island, New York, USA), all experimental steps are based on the manufacturer's instructions. A flow chart for reverse transcription procedure was illustrated in Appendix B.

In brief, for each RT reaction, 10 μg of total RNA and 1 μg of Oligo (dT) primer (0.5 μg/ml) were dissolved in 12 μl of RNase free water. The mixture was incubated at 70 °C for 10 minutes and in ice for another 5 minutes. Then the sample was mixed with 2 μl of 10x PCR buffer, 2 μl of 25 mM $MgCl_2$, 2 μl of 0.1 M DTT and 1 μl of 10 mM deoxynucleoside 5'-triphosphate (dNTP) mixture which comprised dATP, dCTP, dGTP and dTTP. The mixture was then incubated at 42 °C for 5 minutes. Then, 1 μl of reverse transcriptase (Superscript II) enzymes was added and incubated at 42 °C for 50 minutes and 70 °C for another 15 minutes. Finally, 1 μl of RNase H was added into the mixture to digest the remaining RNA sample at 37 °C for 20 minutes. The first strand of cDNA sample was obtained and stored at -20°C until running polymerase chain reaction (PCR).

2.4.2.4 Primers used for the Polymerase Chain Reaction (PCR)

The presence of CFTR, α-ENaC, β-ENaC and γ-ENaC in the endometrial epithelial cells was tested by specially designed mouse CFTR and ENaC cDNA

sequences. The specific Oligo nucleotide primers for GAPDH were: ACC ACA GTC CAT GCC ATG AC (sense) and TCC ACC ACC CTG TTG CTG TA (antisense), corresponding to nucleotides 566 – 1017 with expected cDNA of 451bp (Sabath et al 1990). The specific Oligo primers for CFTR were: CAT CTT TGG TGT TTC CTA TGA TG (sense) and GTA AGG TCT CAG TTA GAA TTG AA (antisense), corresponding to nucleotides 1655 – 2135 with expected cDNA of 481bp (Yorifuji et al 1991). The specific Oligo primers for α-ENaC were: TCA CTT CAG CAC ATC TTC CAC AGC TGC (sense) and GTA TCT GCC TAC CTG GTC CAA GTG GGA (antisense), corresponding to nucleotides 2171-2960 with expected cDNA of 790bp (Ahn et al 1999). The specific Oligo primers for β-ENaC were: CCC CAC CCA GCA ACT AGT GAA CTC AAA (sense) and AAA GCA CGT GTT CCC CTT TCA AGA CTT (antisense), corresponding to nucleotides 1961-2380 with expected cDNA of 420bp (Ahn et al 1999). The specific Oligo primers for γ-ENaC were: GAC TCT CTT CCT GAC ACA AAT GGT CCT (sense) and ACA CAC ATT CTC ACA CAT ACA CAT ACT (antisense), corresponding to nucleotides 2070-2793 with expected cDNA of 724bp (Ahn et al 1999).

2.4.2.5 General procedures of PCR and Competitive Reverse-transcription polymerase chain reaction (RT-PCR)

The polymerase chain reaction (PCR) is an *in vitro* method for amplifying specific DNA sequences. Starting with trace amount of a particular nucleic acid sequence from any source, PCR enzymatically generates millions or billions of exact

copies, thereby making genetic analysis of tiny samples a relatively simple process. PCR was invented by Kary Mullis of Cetus Coporation in 1983.

Theoretically, PCR involves a repetitive series of temperature cycles with each cycle comprising three stages: denaturation of the template DNA in order to separate the strands of the target molecule, then cooling to allow annealing to the template of single-stranded oligonucleotide primers which are specifically designed to flank the region of DNA of interest, and finally, extension of the primers by DNA polymerase. Thus one cycle of PCR doubles the number of target DNA molecules.

In the current study, PCR was used to confirm the expression of CFTR and ENaC in endometrial epithelial cells. And semi-quantitative RT-PCR was used to compare relatively the amount of target DNA molecules using internal standard (GAPDH).

The protocol of PCR analysis was listed in Appendix C. In brief, 1 μl of cDNA template from RT reaction was mixed with 2.5 μl of 10x PCR buffer, 0.5 μl of 0.2 mM dNTP mixture, 2.5 μl of 250 nM of each sense and antisense primers, 0.2 μl of 0.5 U Taq DNA polymerase (Amersham Pharmacia Biotech, Uppsala, Sweden) and DEPC-treated water up to a total of 25 μl. A drop of silicone oil was finally added to the top of the mixture to prevent loss of samples by evaporation whereas the sample was heated up to 94°C during denaturation in PCR reaction.

PCR amplification was performed as follows: denaturation at 94°C for 45 seconds; annealing at different temperature according to different primers used for 1 minute (The annealing temperature of GAPDH, CFTR, α-ENaC, β-ENaC and γ-ENaC were 53 °C, 58 °C, 62 °C, 53 °C and 59 °C respectively.); extension at 72 °C for 1 minute; for 35 cycles and enhancement at 72 °C for 15 minutes. The table shows the sequences and conditions of each primer (Appendix D). Amplifications were performed on a PTC-100 ™ programmable thermal controller (M. J. Res. Inc., Waterown MA, USA). The concentration of cDNA obtained was measured using S2000 UV/VIS spectrophotometer (PerkinElmer MBA2000). The concentration of cDNA was calculated as follows:

[DNA] = Optical density (OD) x Dilution factor x 50

where 50 is the constant for DNA analysis at 260 nm. The PCR products were analyzed by loading 6 μl sample on a 2% agarose gel using one 100-bp marker (GIBCO-BRL, Grand Island, New York, USA).

Semi-quantitative RT-PCR was similar to the traditional RT-PCR but the intensities of the bands of CFTR, α-ENaC, β-ENaC and γ-ENaC were normalized to the GADPH band which was amplified simultaneously (Huang et al 1998).

2.4.2.6 DNA Gel Electrophoresis

DNA gel (2%) was prepared by mixing 1.0 g agarose with 50 ml 1x TAE and boiling to dissolve agarose. Ethidium bromide (1 mg/ml), 15 μl, was added to the mixture before the solidification of the gel. The gel was set in a 8-well or 14-well gel

setter. Then, it was put in a gel tank containing sufficient 1x TAE running buffer. The volume ratio of DNA sample and DNA loading buffer added into the each well was adjusted to 4:1. The gel tank was connected to the power supply, BioRad Laboratories (Alfred Nobel Drive, USA). DNA nucleotide carried negative charges would migrate to the anode in the presence of electrical field. The running time was between 45 to 65 minutes for each experiment. The bands were visualized under UV radiation. Finally, photographs were taken as hard copy.

2.4.3 Capillary Electrophoresis – Laser Induced Fluorescence (CE-LIF)

Capillary Electrophoresis (CE) is an innovative tool for analysis in many areas, including analytical biochemistry, molecular biology, analytical chemistry, medical and other life sciences. Its high sensitivity and analytical specificity provide information on substance's identity, quantity, and purity.

In our experiment, CE was introduced as a tool for rapid and sensitive dsDNA analysis. Basic CE is composed of (1) buffer-filled narrow bore capillaries at high voltage and (2) a detection system. In general, trace amount of samples with fluorescent tag was introduced into the capillaries and was driven along the capillary electrophoretically with high voltage. The velocity at which a charged substance moves per unit of electric field is called its electrophoretic mobilities. This characteristic is determined by its mass various fraction in samples is depend on their different electrophoretic. Laser induced fluorescence is used for detecting the fraction samples. The schematic of the BioFocus capillary electrophoresis systems was shown

in Figure 2.5.

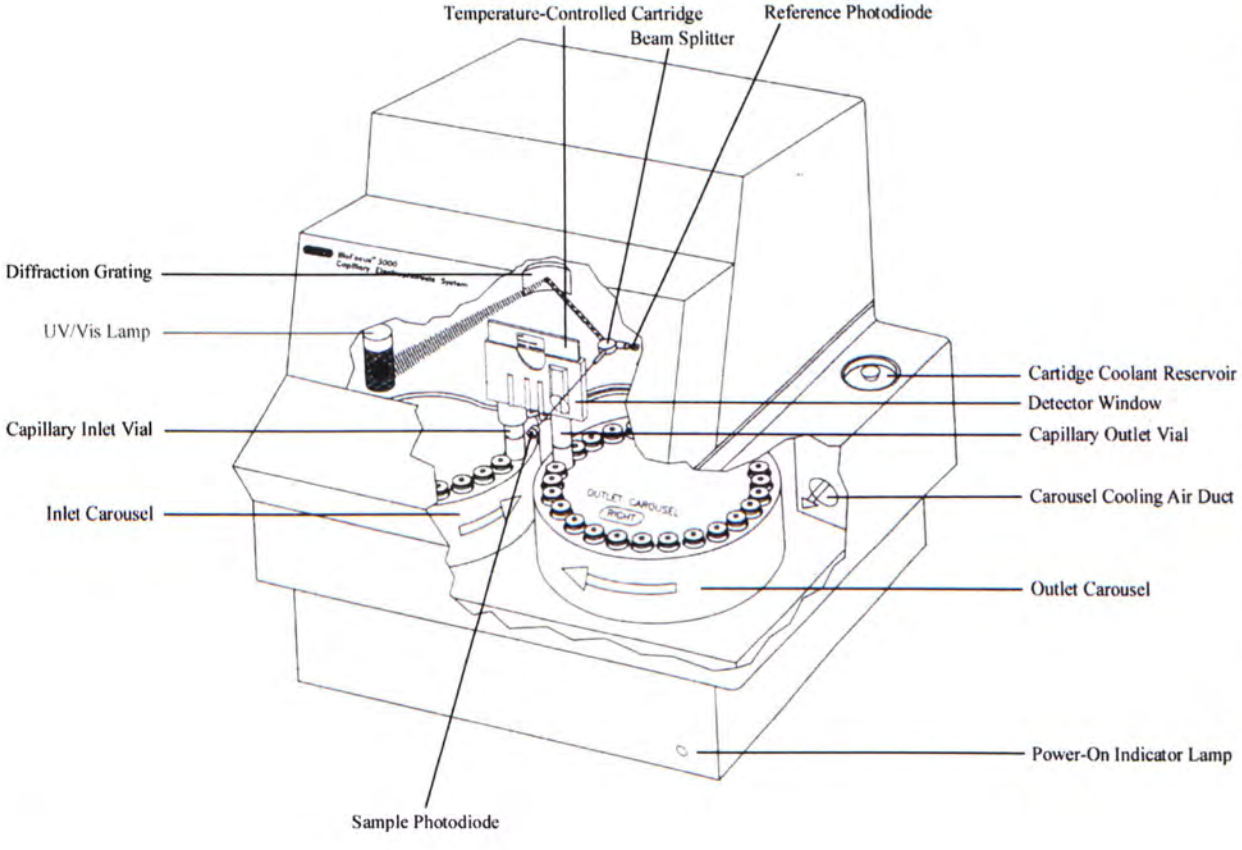


Fig 2.5 BioFocus Capillary Electrophoresis Systems

2.4.3.1 Capillary tube

A fused silica capillary tube is used as a separation vessel in capillary electrophoresis. This silica is used because of its transparency to UV light, making on-line detection possible. The capillary is manufactured with an external polymer coating, which allows the capillary to be manipulated without breaking. The inside diameter of the capillary is important for samples to pass through and small enough to prevent convective mixing and to allow efficient dissipation of Joule heat. The smaller the inside diameter the higher the resolution of the separation and the lower the capillary current and corresponding Joule heating. On the other hand, the lumen must be wide enough to allow the capillary to be filled with various solutions and to provide sufficient path length for the detection of the sample. Capillary electrophoresis systems achieve an optimal balance of these competing factors by using capillaries with 25μm to 75μm inside diameters.

The inside wall of the fused silica capillary tube becomes negatively charged when filled with buffer. This charged surface creates two important effects: electroendosmotic flow of the buffer solution in the capillary, and adsorption of positively-charged sample components to that surface. When sample adsorbs to the surface it covers some of the charged sites and changes the rate of electroendosmotic flow. Adsorption therefore changes the amount of sample available to be detected and the migration time of that sample. Efforts to control or reduce these effects include coating the inside wall of the capillary and putting additives in the buffer.

2.4.3.2 Detection System

Laser-induced fluorescence (LIF) detector was used in our experiment. LIF is highly sensitive and highly increase nucleic acid sensitivity by a factor of 1000 compared to UV detector. LIP is composed of argon ion laser and PMT detector. The argon ion laser emits 3.5 mV at wavelength of 488 nm so as to excite the SYBR Green® dye (fluorescent tag) attached to the nucleic acids. Optical band pass filters was used to reject 488nm excitation light. Which allow 520 nm light emitted by SYBR Green® to pass through. The intensity of 520 nm light was detected by PMT detector and the signal was represented by the computer.

2.4.3.3 Experimental Procedure

Aliquots (2 μL) of RT-PCR samples were diluted 1:10 with Tris-EDTA (pH 8.0) prior to analysis by CE-LIF. A BioFocus® CE-LIF (BioRad Laboratories, Alfred Nobel Drive, USA) equipped with an argon ion LIF detector and controlled by BioFocus 3000 software (version 6.0) was used for the analysis of RT-PCR samples. Excitation of the intercalating dye was at 488 nm and emission was detected at 520 nm. The instrument was configured for reversed polarity (i.e., cathode on the inlet side). A BioCAP coated capillary (Cat. No. 148-3084, Bio-Rad Laboratories, Alfred Nobel Drive, USA) with a 75 μm ID, 375 μm OD, and length of 50 cm was housed in a BioFocus capillary cartridge (BioRad Laboratories, Alfred Nobel Drive, USA) and maintained at 22 ˚C. The CE dsDNA run buffer contains 2x TBE. Immediately prior to use, an appropriate amount of intercalating dye 20 μL of SYBR® Green (fluorescent

tag) was added to 10 mL of run buffer, which was mixed for at least 2 minutes. The sample injection/run routine was as follows: 1) The 45 second, high pressure preparation cycle (Prep cycle 1) fills the capillary with fresh run buffer at the beginning of each analysis. The 0 second cycles (Prep cycle 2 and 3) dip the capillary and electrode into vials containing deionized water to rinse their surfaces to prevent buffer carry-over into the sample vial. 2) RT-PCR samples (diluted 1:10 in Tris-EDTA, 20 μL total volume) were subjected to electokinetic loading at 10 kV for 2 seconds. 3) Separation was performed at a field strength of 273V/cm (+4.5kV) for 30 min. At the beginning and end of each day of use, the capillary was regenerated by rinsing consecutively with water and nitrogen gas for 5 min each.

2.4.3.4 Data Analysis

Inside LIF^2 Detector, when sample component passes through the capillary's detection window, the PMT generates a signal proportional to the amount of light emitted. The computer normalizes this signal based on each PMT's sensitivity and gain setting. The computer also filters the signal and adds an autozero offset. The computer collects a database representing signal amplitude versus time during the sample run, which can be seen in real time in an electropherogram displayed on the computer's monitor during the run. After the run, file data may be viewed and further analyzed using Bio-Rad Integration software.

2.4.4 Statistical Analysis

All results were expressed as mean ± SEM. Comparisons between groups of data were carried out using student's unpaired *t*-test. A *P* value less than 0.05 was considered to be significant.

Chapter 3: Results

3.1 Influence of Ovarian Hormones on Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) and Epithelial Na^+ Channel (ENaC) Expression in Mouse Endometrial Epithelium

Summary

Previous studies have demonstrated amiloride-sensitive Na^+ absorption, presumably mediated by epithelial sodium channel (ENaC), under basal condition and CFTR-mediated Cl^- secretion upon neurohormonal stimulation in the mouse endometrial epithelium. However, the roles of ovarian hormones (estrogen and progesterone) in regulating CFTR and ENaC in mouse endometrial epithelium are poorly understood. The present study investigated the functional expression of ENaC and CFTR upon different concentrations of estrogen and progesterone stimulation *in vivo* using semi-quantitative RT-PCR analysis and *in vitro* using short-circuit current (I_{SC}) technique. The data showed that CFTR and ENaC were differentially expressed throughout the estrus cycle with CFTR maximally expressed at proestrus and estrus, while all ENaC subunits (α, β and γ) were abundantly detected at metestrus and diestrus. In ovariectomized mice, injection of 17β-estradiol increased CFTR mRNA level and decreased γ-ENaC mRNA level after 24-hour treatment as compared to control. In contrast, injection of progesterone decreased CFTR and increased α-ENaC

and γ-ENaC mRNA level after 48 hours treatment. To further explore the functional roles of estrogen and progesterone played in regulating CFTR and ENaC expression, CFTR and ENaC activities were assessed using the I_{SC} technique in 17β-estrodial and/or progesterone treated primary cultured mouse endometrial epithelial monolayers. The amiloride-sensitive I_{SC}, mediated by ENaC, was significantly reduced in 0.1μM 17β-estradiol-treated epithelia from 17.87 ± 1.04 μA/cm^2 (control) to 13.92 ± 0.92 μA/cm^2 ($P<0.05$, n=6). However, the forskolin-activated I_{SC}, presumably mediated by CFTR, was significantly enhanced in epithelia treated with 0.01 μM 17β-estradiol, from 5.1 ± 0.1 μA/cm^2 (control) to 8.3 ± 0.7 μA/cm^2 ($P<0.05$, n=6). In contrast, 0.1μM progesterone significantly reduced the forskolin-induced I_{SC} from 10.21 ± 0.56 μA/cm^2 (control) to 8.76 ± 0.60 μA/cm^2 ($P<0.05$, n=6). Taken together, our data indicated that CFTR and ENaC are differentially regulated by estrogen and progesterone both *in vivo* and *in vitro*.

The endometrium of the uterus provides an optimal microfluid environment for sperm movement and capacitation, successful implantation and development of the blastocyst. Its absorptive and secretory activities are under constant influence of ovarian hormones (estrogen and progesterone). Uterine fluid movements across epithelia are secondary to the movements of solutes, mainly electrolytes. Na^+ and Cl^- conductance are essential for the electrolyte and fluid absorption and secretion, respectively. Active Na^+ absorption drives Cl^- and fluid out of the lumen into blood, while active Cl^- secretion drives Na^+ and fluid from the plasma into the lumen. Previous studies have demonstrated amiloride-sensitive Na^+ absorption and CFTR-mediated Cl^- secretion in the mouse endometrial epithelium. Evidence suggested that ENaC and CFTR are possibly involved in mediating the absorptive and secretory activities, respectively, of the endometrium (Chan et al 2000b, Chan et al 2000a, Deachapunya & O'Grady 1998). However, the regulation of these ion channels by ovarian hormones has not been elucidated.

In order to understand the influence of ovarian hormones (estrogen and progesterone) on ENaC and CFTR in mouse endometrial epithelia and thus their effect on uterine fluid environment, the present studies investigated the mRNA expression of CFTR and ENaC throughout estrus cycle *in vivo* using semi-quantitative RT-PCR technique and the effect of estrogen and progesterone on ENaC and CFTR activities *in*

vitro using short-circuit current (I_{SC}) technique. The results indicate differential expression of ENaC and CFTR during the estrus cycle and under different hormonal treatment. Interaction between ENaC and CFTR is also suggested by their co-expression in the mouse endometrial epithelia for the maintenance of a suitable fluid environment.

Results

RT-PCR demonstration of co-expression of CFTR and ENaC

The co-expression of CFTR and ENaC in the mouse uterus was demonstrated by RT-PCR (Fig 3.1.1). Using primers designed from mouse sequences for CFTR, α-, β-, and γ-ENaC subunits, bands at 481, 790, 420 and 724 bp as expected for CFTR, α-, β-, and γ-ENaC subunits, respectively, were obtained (Fig 3.1.1). As shown in figure 3.1.1a, T84 cell line, a well-known cell line expresses CFTR gene, was used as a positive control for CFTR. Total RNA from the mouse kidney, known to express the three ENaC subunits, was used as a positive control for ENaC (figure 3.1.1b). The absence of DNA template was served as a negative control in both agarose gel experiments. The identity of each PCR product was determined using sequencing technique. The sequence of CFTR PCR fragment showed 98% homology to that previously published for the mouse CFTR (nucleotide positions 1655 – 2135 base pairs, gene bank accession number M60493) (Yorifuji et al 1991). The sequence of the fragments amplified for the α-ENaC, β-ENaC and γ-ENaC subunits showed 98%, 99% and 99% homology to these previously published for the mouse α-ENaC (nucleotide positions 2171-2960 base pairs, accession number AF112185), β-ENaC (nucleotide positions 1961-2380 base pairs, accession number AF112186) and γ-ENaC (nucleotide positions 2070-2793 base pairs, accession number AF112187), respectively (Ahn et al 1999). The mouse GAPDH amplified sequence was identical to that previously reported (Sabath et al 1990).

Expression patterns of CFTR and ENaC throughout the estrus cycle

To study the expression pattern of CFTR and ENaC subunits during the estrus cycle, adult female mice were synchronized by injecting 5 μg/100g (i.p.) 17β-estradiol two days before experiment. The stages of estrus cycle were then identified by examining the morphological changes of the vagina. The expression levels of CFTR and ENaC at different stages of estrus cycle were demonstrated by Semi-quantitative RT-PCR with GAPDH used as an internal marker.

Figure 3.1.2 shows CFTR mRNA expression level during the estrus cycle. As shown in Fig 3.1.2a, the upper bands are DNA fragments corresponding to 481 bp CFTR and the lower bands are GAPDH at 340 bp. The intensity of GAPDH in each sample was normalized and amplified with the target DNA fragment simultaneously. The expression level of CFTR was measured by the relative ratio of CFTR to that of GAPDH. CFTR mRNA was detected at proestrus and early estrus and reached a maximum at estrus but was not detectable at metestrus and diestrus (Fig 3.1.2a). Figure 3.1.2b is the intensity ratio of CFTR to GAPDH plotted against estrus cycle.

Figure 3.1.3 shows the α-ENaC mRNA expression level during the estrus cycle. As shown in Figure 3.1.3a, the upper bands are DNA fragment corresponding to 790 bp α-ENaC and the lower bands are that of GAPDH at 340 bp. α-ENaC mRNA expression level was low at proestrus and estrus and was dramatically increased at metestrus with slightly decreased at diestrus (Fig 3.1.3a). Figure 3.1.3b is the intensity ratio of α-ENaC to GAPDH plotted against estrus cycle.

Figure 3.1.4 shows the β-ENaC mRNA expression level during the estrus cycle.

As shown in Figure 3.1.4a, the upper bands are DNA fragment correspond to 420 bp β-ENaC and the lower bands are that of GAPDH at 340 bp. The β-ENaC mRNA expression level was slightly increased from proestrus to estrus and continuously increased at metestrus and diestrus (Fig 3.1.4a). Figure 3.1.4b is the intensity ratio of β-ENaC to GAPDH plotted against estrus cycle.

Figure 3.1.5 shows the γ-ENaC mRNA expression level during the estrus cycle. As shown in Figure 3.1.5a, the upper bands are DNA fragment correspond to 724 bp γ-ENaC and the lower bands are that of GAPDH at 340 bp. The γ-ENaC mRNA expression was low from proestrus to metestrus but suddenly increased at diestrus (Fig 3.1.5a). Figure 3.1.5b is the intensity ratio of γ-ENaC to GAPDH plotted against estrus cycle.

Regulation of CFTR and ENaC expression by ovarian hormone

To confirm the variation in CFTR and ENaC expression during the estrus cycle was due to the influence of ovarian hormones, experiments were conducted in ovariectomized mice with injection of either 2 μg/100g (i.p.) 17β-estradiol or 1.5 mg/100g (i.p.) progesterone.

The semi-quantitative RT-PCR results showed products with expected sizes corresponding to cDNA for the GAPDH (451 bp), the CFTR (481 bp), the α-ENaC subunit (790 bp), the β-ENaC subunit (420 bp) and γ-ENaC subunit (724 bp) (Fig 3.1.6 and Fig 3.1.7). Semi-quantitative RT-PCR also showed the relative abundance of CFTR, α-, β- and γ-ENaC mRNAs in different hormonal treatment after normalization

to the expression level of GAPDH which was used as an internal marker (Table 3.1.1).

After 24 hours hormonal treatment, as shown in figure 3.1.6, the expression level of the CFTR mRNA was increased in both progesterone and 17β-estradiol treatment as compared to that of control. Injection of 17β-estradiol alone had a higher expression level of CFTR mRNA as compared with progesterone treatment. The α-ENaC and β-ENaC subunits mRNA signals of hormone-injected group had no difference from that of control. The expression level of the γ-ENaC subunit mRNA was lower in 17β-estradiol treatment as compared with control, but there was no difference in progesterone treatment.

Figure 3.1.7 shows the mRNA expression level of CFTR, α-, β-, and γ-ENaC after 48 hours hormonal treatment. The expression level of CFTR mRNA was decreased in progesterone treatment and increased in 17β-estradiol treatment as compared with control. The α-ENaC subunit mRNA signal was increased in progesterone treatment but had no difference in 17β-estradiol treatment. The β-ENaC subunits mRNA signals had no difference as compared with control. The expression level of the γ-ENaC subunit mRNA was higher in progesterone treatment than in 17β-estradiol treatment.

Table 3.1.1 summarize the mRNA expression level of CFTR, α-, β- and γ-ENaC in ovariectomized ICR mice with intraperitoneal administration of 17β-estradiol and progesterone.

Effect of 17β-estradiol and progesterone on I_{SC}

Different concentrations of 17β-estradiol and progesterone were added basolaterally and apically 24 hours prior to the experiment. In 0.1 μM 17β-estradiol-treated epithelia, the basal I_{SC} and amiloride-sensitive I_{SC} were significantly reduced from 18.46 ± 0.90 μA/cm^2 to 13.91 ± 0.83 μA/cm^2 (n=6, P<0.05) and from 17.87 ± 1.04 μA/cm^2 to 13.92 ± 0.92 μA/cm^2 (n=6, P<0.05), respectively (Fig 3.1.8). 0.01 μM of 17β-estradiol enhanced the forskolin-induced Isc (8.3 ± 0.7 μA/cm^2 as compared to control 5.1 ± 0.1 μA/cm^2, n=6, P<0.05), but did not affect the amiloride-sensitive I_{SC} (n=6) (Fig 3.1.9). In 0.1 μM progesterone-treated epithelia, the basal I_{SC} and the forskolin-induced I_{SC} were significantly reduced from 9.77 ± 0.43 μA/cm^2 to 6.89 ± 0.78 μA/cm^2 (n=6, P<0.05) and from 10.21 ± 0.56 μA/cm^2 to 8.76 ± 0.60 μA/cm^2 (n=6, P<0.05), respectively. There was no significant effect of 0.01 μM progesterone on the amiloride-sensitive (n=6) and forskolin-induced I_{SC} (n=6) (Fig 3.1.10). The present results suggest that the expression of CFTR and ENaC in the endometrium could be regulated by both ovarian hormones; however, the two channels may be differentially expressed under different hormonal concentration profiles.

Discussion

The endometrium and the uterine fluid formation are under constant influence of ovarian hormones. Hormonal regulation of the uterine fluid volume has been demonstrated in rat and mice, indicating that estrogen and progesterone increase and decrease uterine fluid volume, respectively (Casslen 1986). However, the molecular entities governing the electrolyte transport that is essential for fluid formation remain poorly understood. Recent studies on primary cultures of mouse endometrial epithelia have demonstrated a substantial amiloride-sensitive basal short-circuit current, the possible involvement of ENaC (Chan et al 1997b). Moreover, recent electrophysiological studies have demonstrated that CFTR would be the key player in active Cl^- secretion across the female reproductive tract (Chan et al 2000b, Chan et al 1999, Deachapunya & O'Grady 1998).

The present finding of co-expression of CFTR and ENaC throughout the estrus cycle suggests that they may play secretory and reabsorptive roles in the uterine fluid formation respectively. The expression of CFTR mRNA at proestrus and estrus is expected to bring about higher rate of Cl^- secretion and therefore greater fluid accumulation. On the contrary, the maximal level of all ENaC mRNA expression detected at metestrus and diestrus is expected to absorb Na^+ and water, thus decreases fluid accumulation. These secretory and absorptive activities of the uterus, presumably mediated by CFTR and ENaC, respectively, are under constant influence

of ovarian hormones (estrogen and progesterone).

The present study has provided evidence for the regulation of CFTR and ENaC expression influenced by different hormonal treatment *in vivo* and *in vitro*. The presently demonstrated co-expression of CFTR and ENaC in cultured endometrial epithelial cells enabled us to investigate possible interaction between the two in a native system since most of the reported studies were performed in expression systems where CFTR and ENaC are exogenously introduced. Previous report has shown that 17β-estradiol induces secretion while progesterone promotes reabsorption of uterine fluid in rats (Clemetson et al 1977). The presently demonstrated maximum expression of CFTR at proestrus and estrus where the concentration of circulating estrogen is high suggests an important role of CFTR in secreting NaCl and fluid in the uterus. On the contrary, all ENaC subunits (α, β and γ) were abundantly detected at metestrus and diestrus where the concentration of progesterone is high indicating an important role of ENaC in Na^+ and water reabsorption in the uterus.

Stimulation of CFTR expression by estrogen both *in vitro* and *in vivo* has been reported earlier in rat uterus (Rochwerger & Buchwald 1993, Rochwerger et al 1994). The present studies have demonstrated functionally the enhancement of the forskolin-induced I_{SC} associated with an increase in CFTR mRNA expression in response to 17β-estradiol administration. Our results also show the suppression of the forskolin-induce I_{SC} associated with a decrease in CFTR mRNA expression in

response to progesterone administration, indicating the down-regulation of CFTR by progesterone.

The present studies demonstrated the decrease of the amiloride-sensitive I_{SC} associated with a decrease in γ-ENaC subunit mRNA expression in response to 17β-estradiol administration and the increase in α- and γ-ENaC subunits mRNA expression in response to progesterone administration. Together with the detection of ENaC in estrus cycle with minimal expression at proestrus and estrus and maximal expression at metestrus and diestrus, the data indicate the down-regulation of ENaC by estrogen and up-regulation by progesterone.

The co-expression of CFTR and ENaC in the uterus provides evidence supporting an ability of the uterus to both secrete and absorb uterine fluid. Long and Evans (Long & Evans 1922) first described the accumulation of fluid which distend the uterine horns of rats at proestrus and estrus and which is absent at diestrus. There was no doubt that the horns could become distended as a result of estrogen stimulation. Later experiment under the influence of both estrogen and progesterone clearly indicated that estrogen stimulated fluid secretion and progesterone stimulated fluid absorption in the rat uterus (Clemetson et al 1977). The present results suggest two different mechanisms possible for the disappearance of uterine fluid at diestrus. First, the down-regulation of CFTR to slow down fluid production and second, the up-regulation of ENaC to increase the rate of reabsorption. The absence of CFTR at

diestrus may further augment ENaC function to facilitate fluid reabsorption considering another role of CFTR as a negative regulator of ENaC (Stutts et al 1995). The co-expression of CFTR and ENaC in the uterus may also be of physiological significance. Maximal CFTR expression at proestrus and estrus may enable higher rate of uterine fluid production to facilitate sperm transport and sperm capacitation, down-regulation of CFTR and up-regulation of ENaC at metestrus and diestrus may reduce the fluid volume in the lumen to enhance close contact between the endometrial surface to facilitate the implantation of embryo.

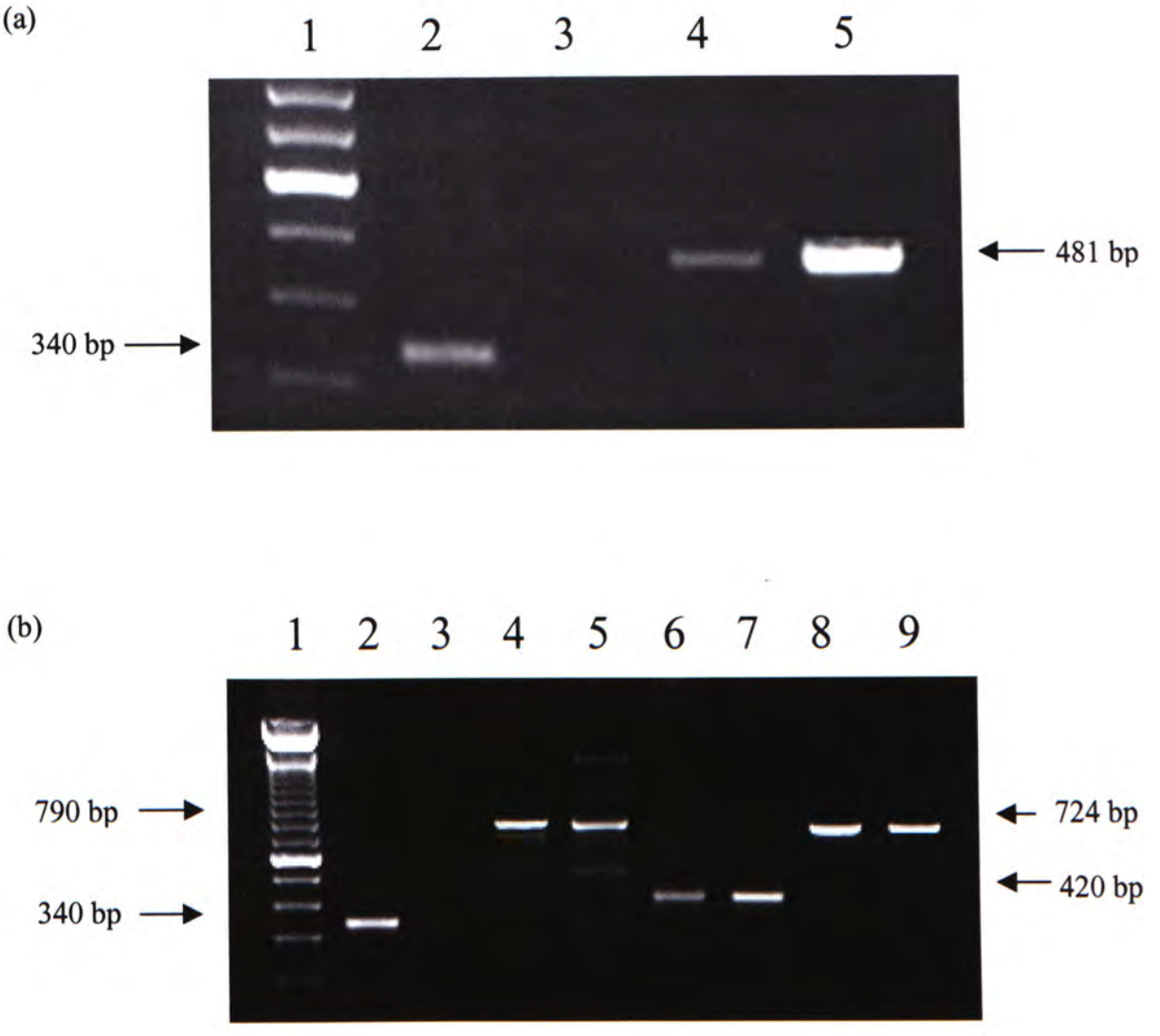
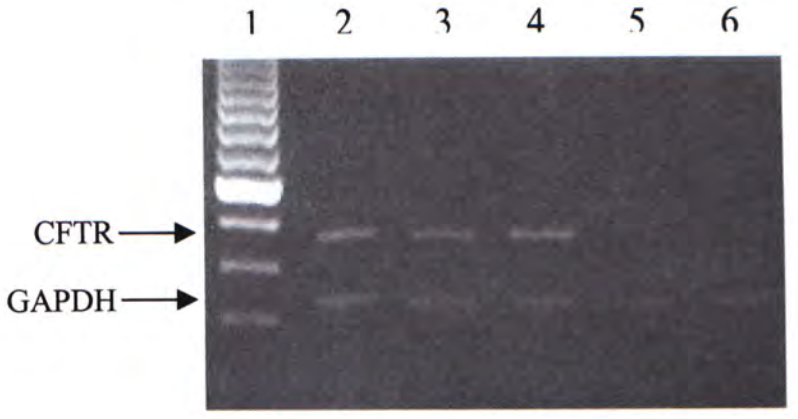


Fig 3.1.1 RT-PCR demonstration of the presence of ENaC and CFTR in the mouse uterus. (a) 100 bp DNA marker in lane 1, GAPDH in lane 2 (340 bp), negative control with no template in lane 3, CFTR of T84 cell line as positive control in lane 4 and endometrial epithelial cells in 5 (481 bp). (b) 100 bp DNA marker in lane 1, GAPDH in lane 2 (340 bp), negative control with no template in lane 3, α- (790 bp), β- (420 bp), and γ-ENaC (724 bp) subunits of kidney as a positive control in lane 4, 6 and 8 respectively and α- (790 bp), β- (420 bp), and γ-ENaC (724 bp) subunits of endometrial epithelial cells in lane 5, 7 and 9 respectively.

(a)



(b)

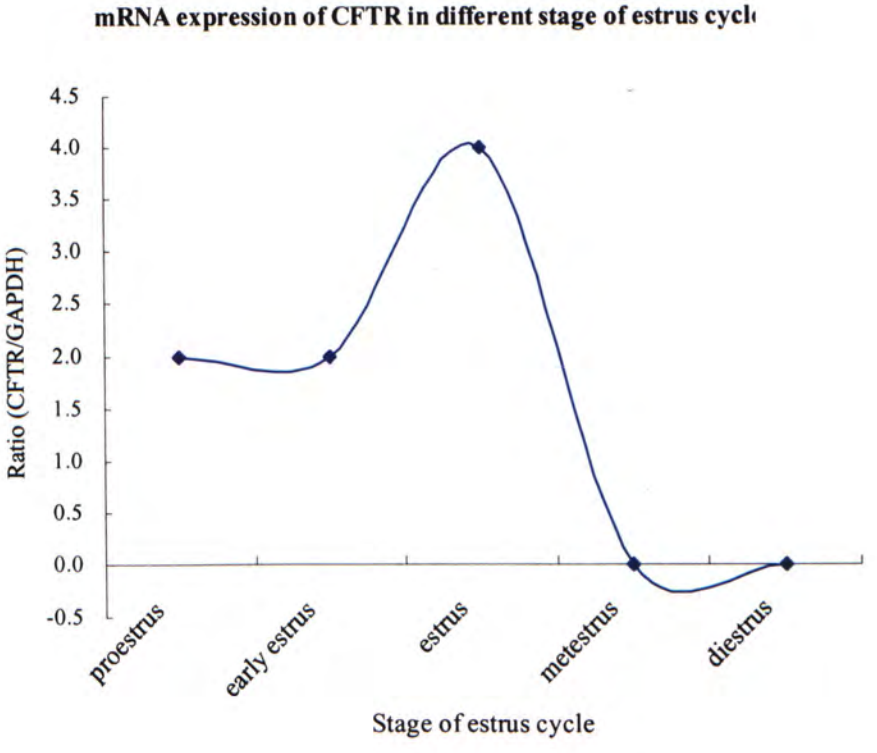
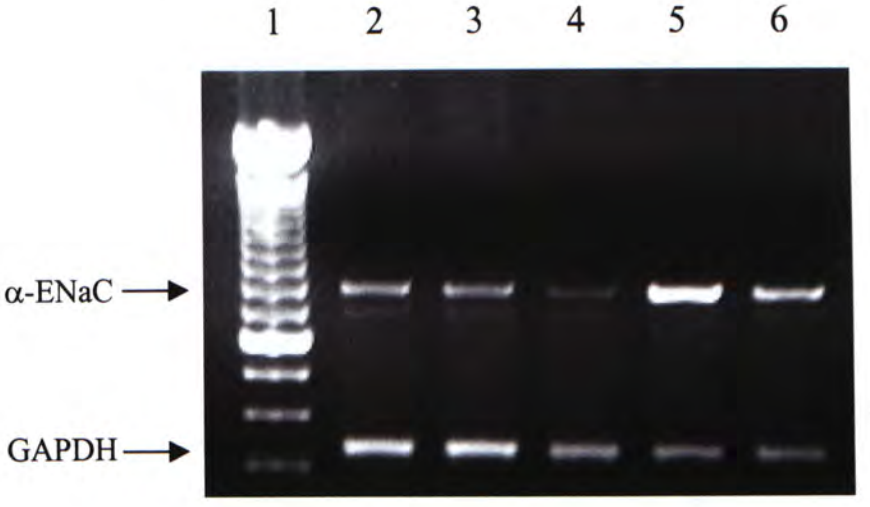


Fig 3.1.2 CFTR mRNA expression throughout estrus cycle. (a) agarose gel showing different expression level of CFTR at proestrus (lane 2), early estrus (lane 3), estrus (lane 4), metestrus (lane 5) and diestrus (lane 6). 100 bp DNA ladder in lane 1. (b) Intensity ratio of CFTR (481 bp) to GAPDH (340 bp) at different stage of estrus cycle.

(a)



(b)

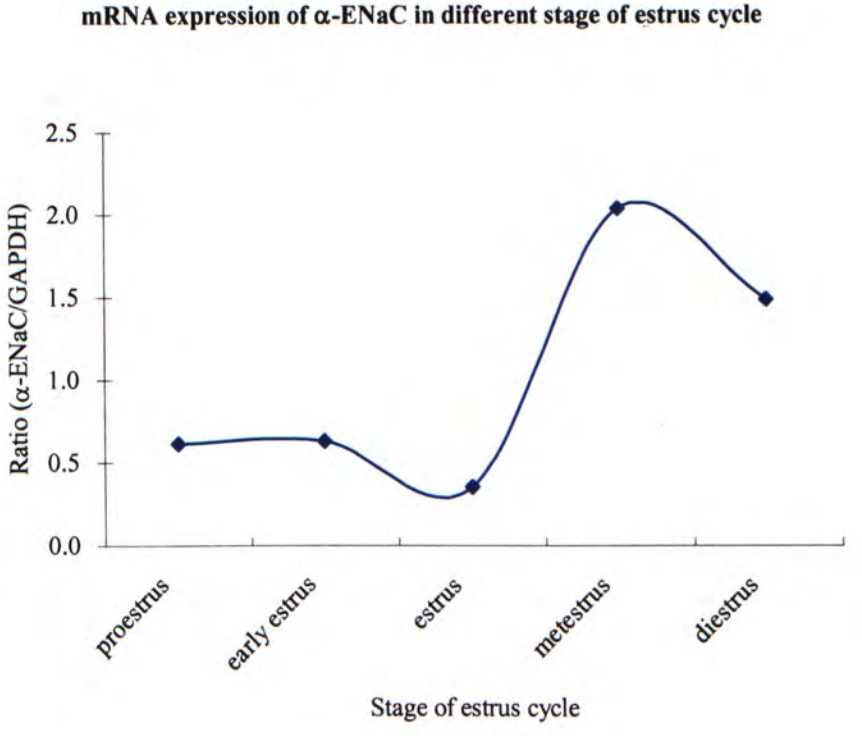
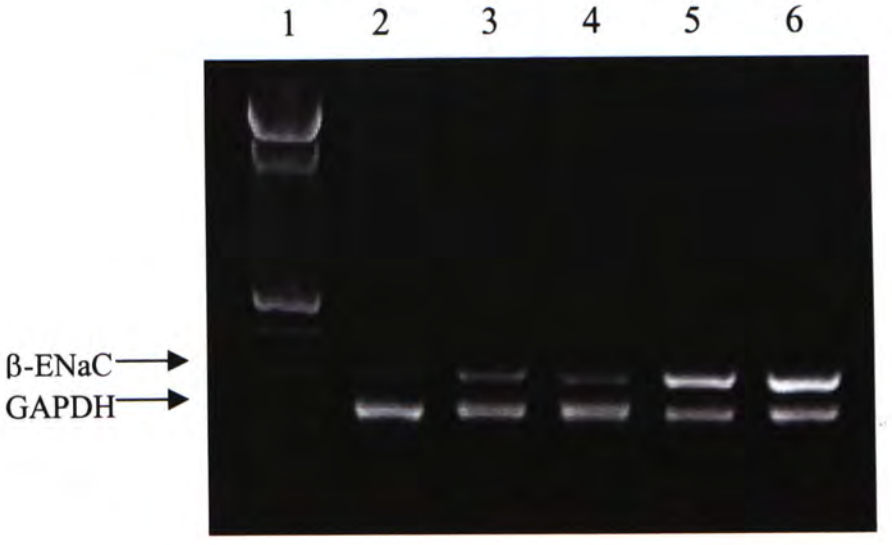


Fig 3.1.3 α-ENaC mRNA expression throughout estrus cycle. (a) agarose gel showing different expression level of α-ENaC at proestrus (lane 2), early estrus (lane 3), estrus (lane 4), metestrus (lane 5) and diestrus (lane 6). 100 bp DNA ladder in lane 1. (b) Intensity ratio of α-ENaC (790 bp) to GAPDH (340 bp) at different stage of estrus cycle.

(a)



(b)

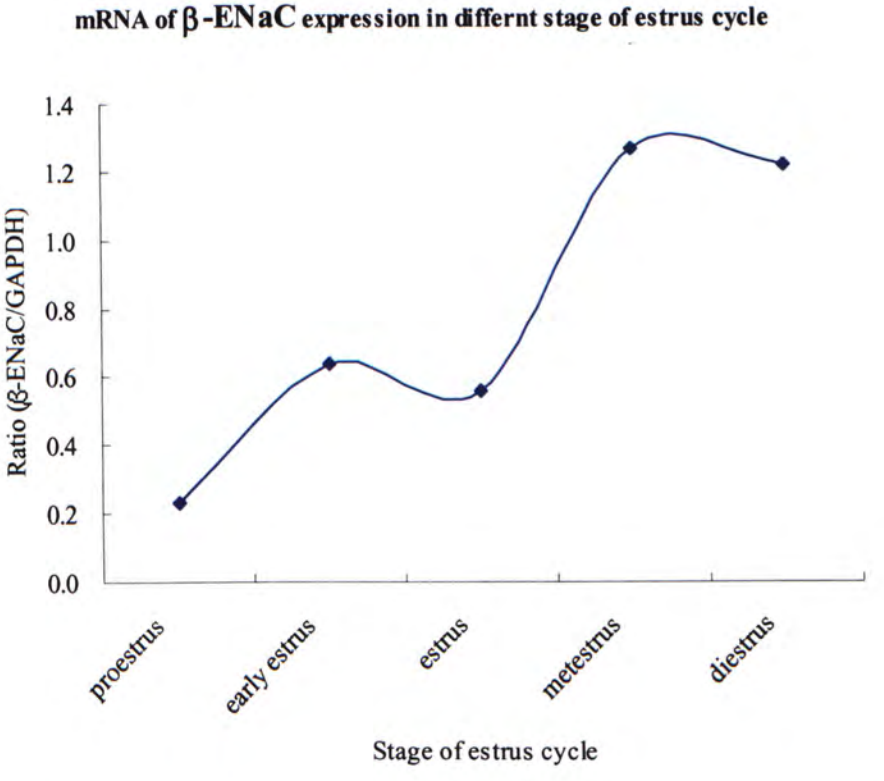
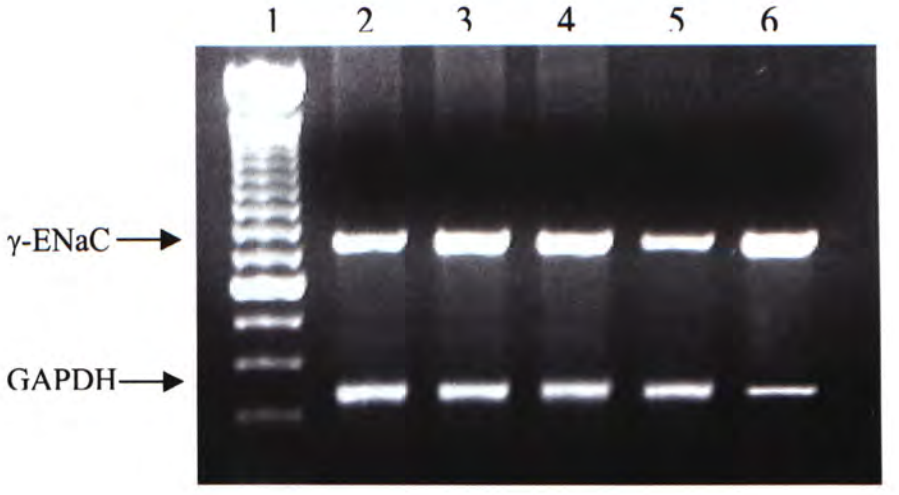


Fig 3.1.4 β-ENaC mRNA expression throughout estrus cycle. (a) agarose gel showing different expression level of β-ENaC at proestrus (lane 2), early estrus (lane 3), estrus (lane 4), metestrus (lane 5) and diestrus (lane 6). 100 bp DNA ladder in lane 1. (b) Intensity ratio of β-ENaC (420 bp) to GAPDH (340 bp) at different stage of estrus cycle.

(a)



(b)

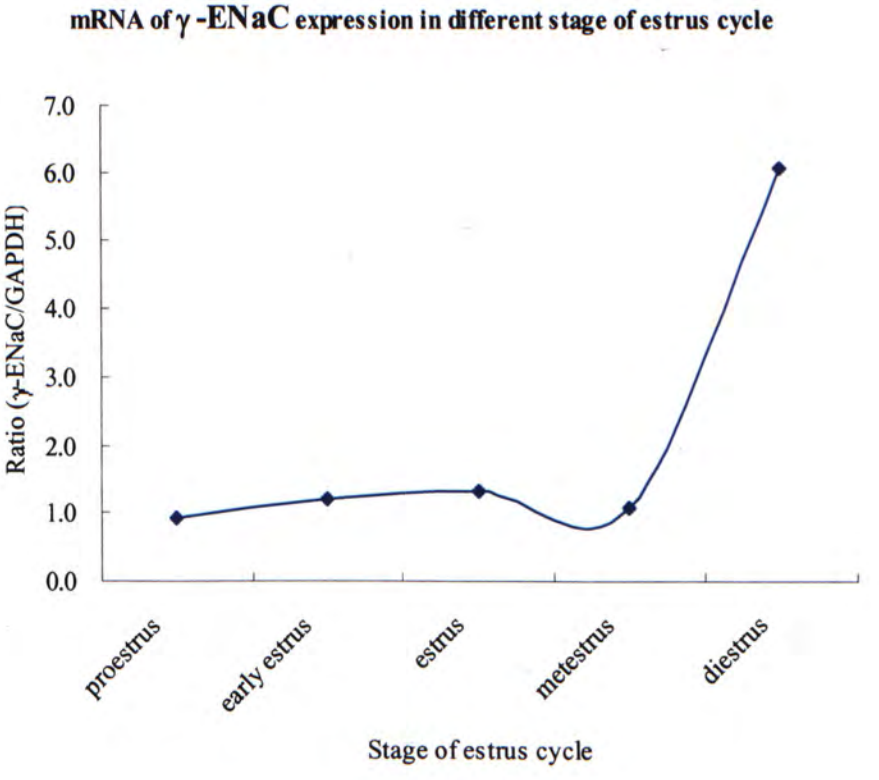


Fig 3.1.5 γ-ENaC mRNA expression throughout estrus cycle. (a) agarose gel showing different expression level of γ-ENaC at proestrus (lane 2), early estrus (lane 3), estrus (lane 4), metestrus (lane 5) and diestrus (lane 6). 100 bp DNA ladder in lane 1. (b) Intensity ratio of γ-ENaC (724 bp) to GAPDH (340 bp) at different stage of estrus cycle.

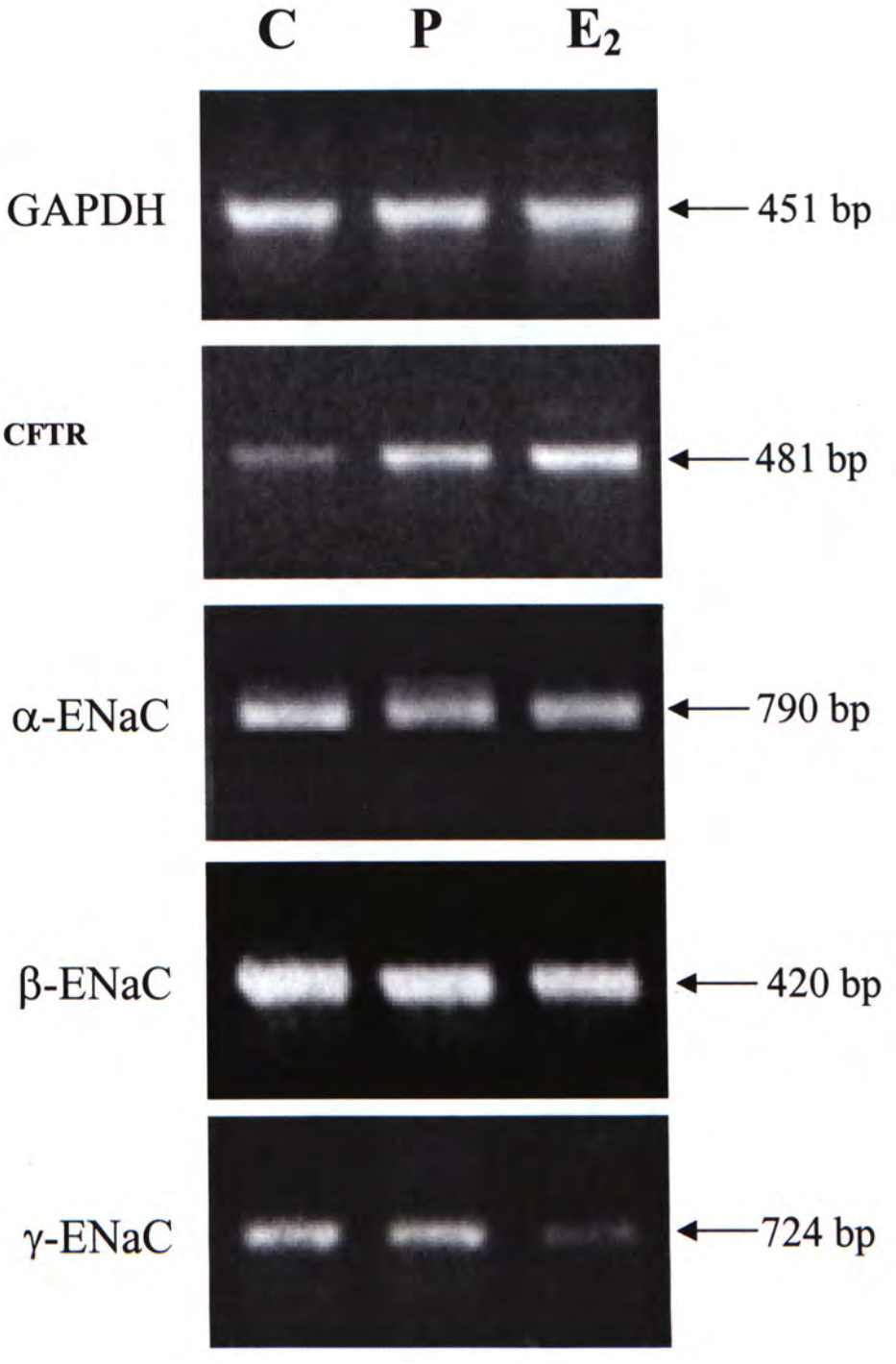


Fig 3.1.6 Effect of progesterone (P) and 17β-estradiol (E_2) on uterine CFTR and all ENaC subunits after 24 hours treatment. Agarose gel showing equal amounts of cDNA, as determined from the intensity of the GAPDH band, with primers specific for CFTR and each of the ENaC subunits. The specific primers are named on the left and their expected sizes are shown on the right.

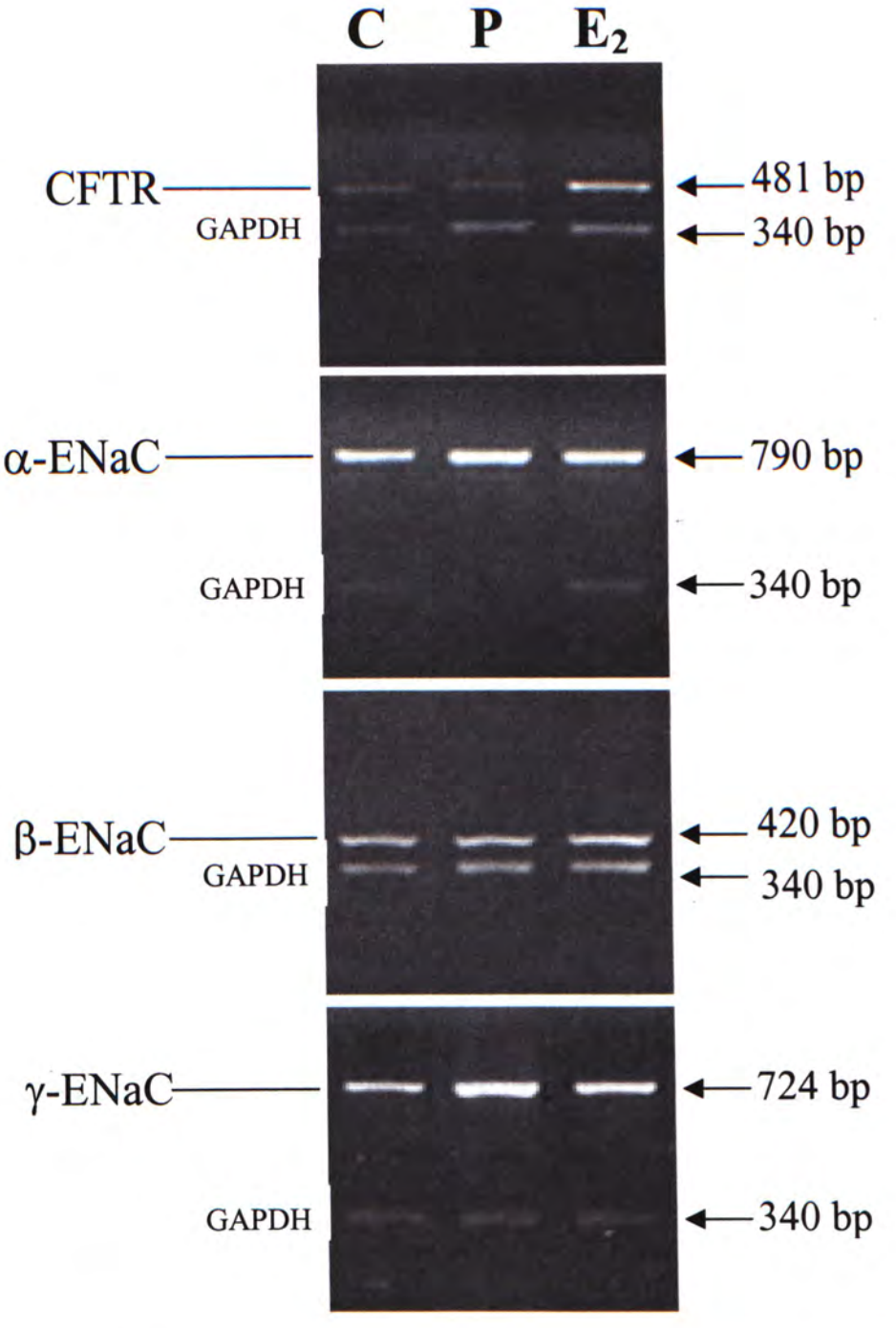


Fig 3.1.7 Effect of progesterone (P) and 17β-estradiol (E_2) on uterine CFTR and all ENaC subunits after 48 hours treatment. The cDNA from uterus were amplified with GAPDH primer and primers specific for CFTR or each of the mouse ENaC subunits simultaneously. The specific primers are named on the left and their expected sizes are shown on the right.

	24 hours		*48 hours*	
	17β-estradiol Treatment	Progesterone Treatment	17β-estradiol Treatment	Progesterone Treatment
CFTR	++	+	+	-
α-ENaC	ND	ND	ND	++
β-ENaC	ND	ND	ND	ND
γ-ENaC	-	ND	+	++

Table 3.1.1 Summary of the mRNA expression level of CFTR, α-, β- and γ-ENaC in ovariectomized ICR mice with intraperitoneal administration of 17β-estraiol (E_2) and progesterone (P). "+" represents an increase in mRNA expression; "++" represents more increase in mRNA expression; "-" represents a decrease in mRNA expression and "ND" represents no difference in mRNA expression as compared to control.

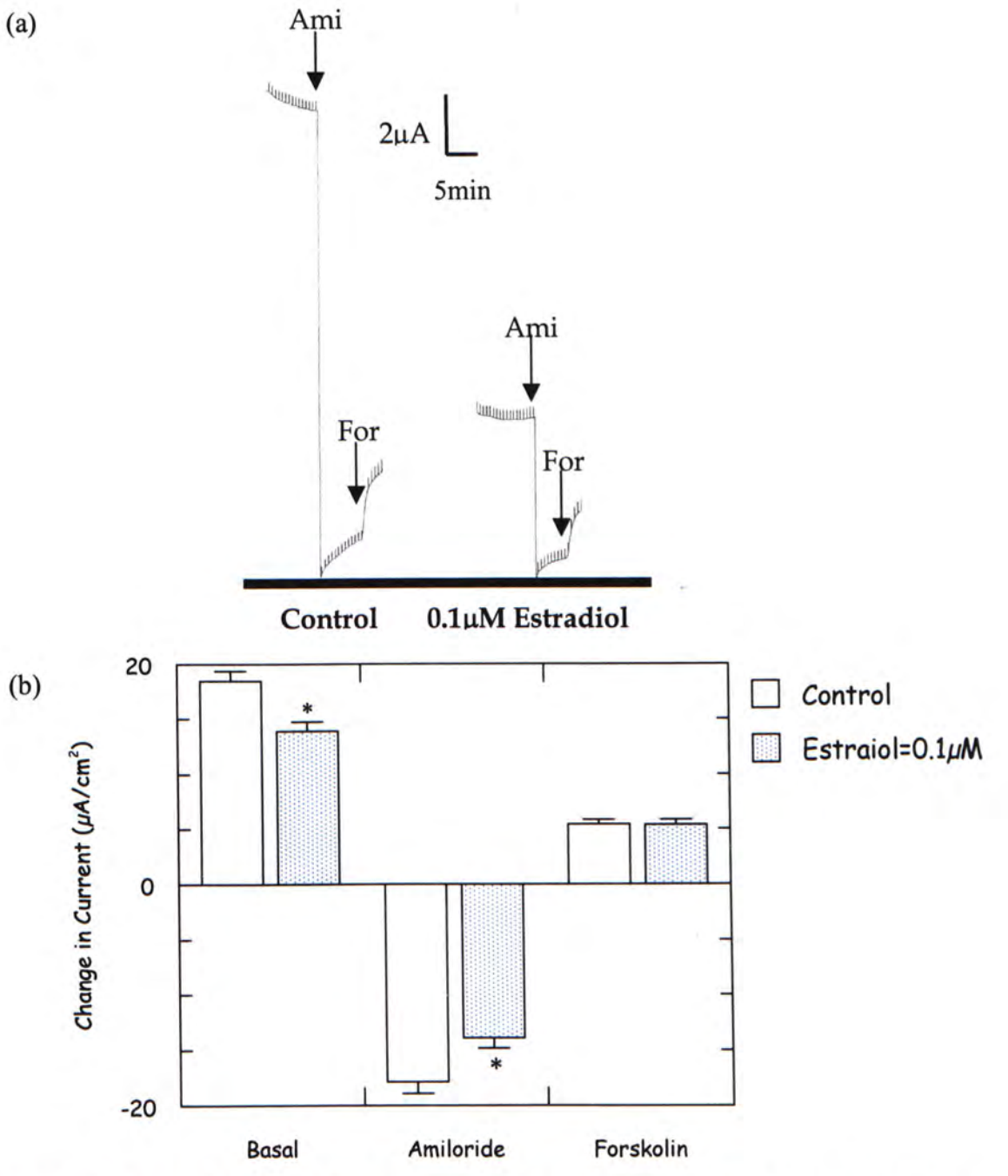


Fig 3.1.8 Effect of 17β-estradiol (0.1 μM) on the amiloride-sensitive I_{SC} upon 24-hour treatment prior to the experiment. (a) Representative I_{SC} recording showing I_{SC} attributed to amiloride-sensitive basal Na^+ absorption and forskolin-induced Cl^- secretion in control and 0.1 μM 17β-estradiol treated epithelium. (b) Statistical results showing the difference between basal, amiloride-sensitive and forskolin-induced I_{SC} in control and 0.1 μM 17β-estradiol treated epithelium. Data are means ± SEM. (*P<0.05, n=6)

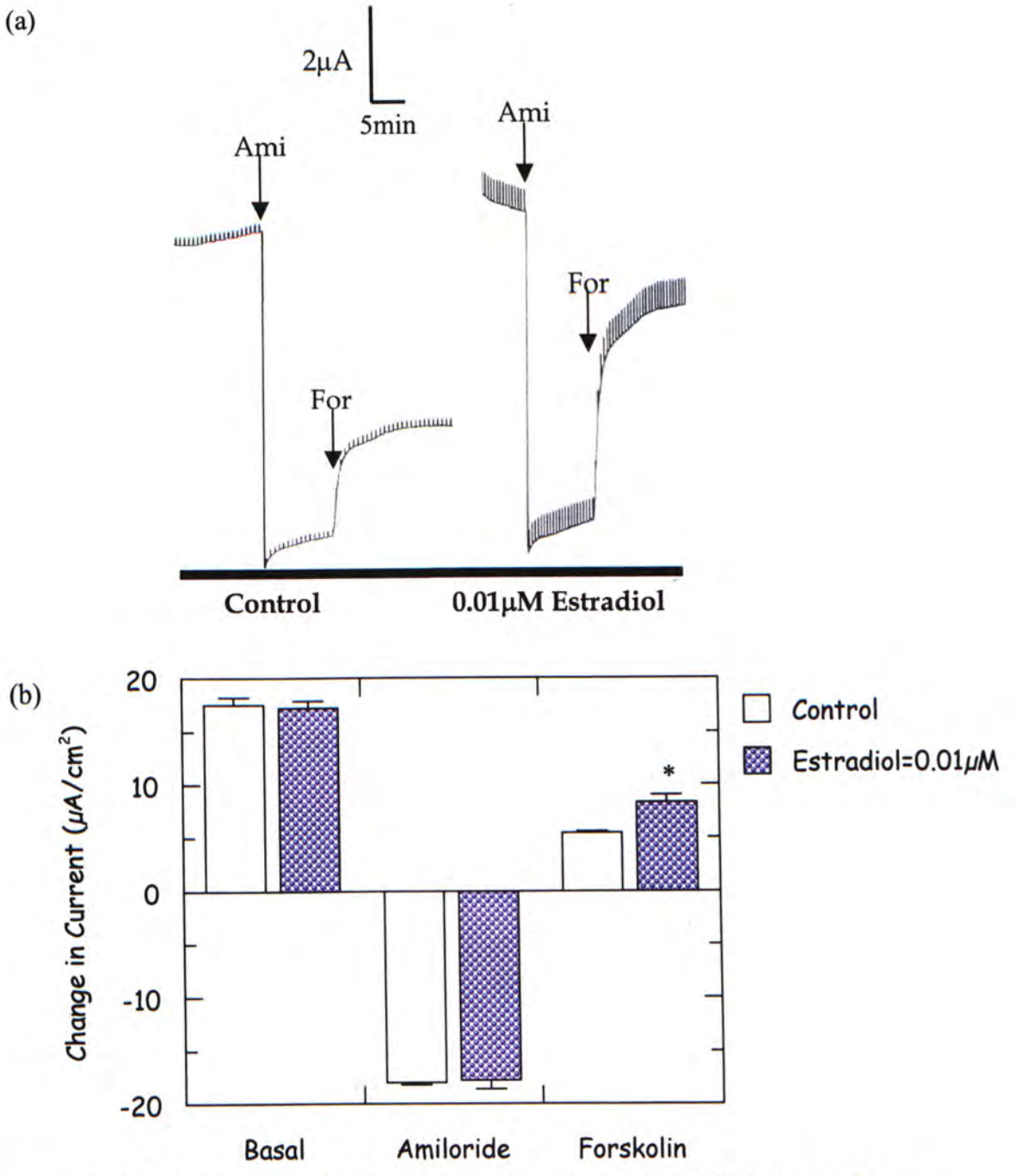
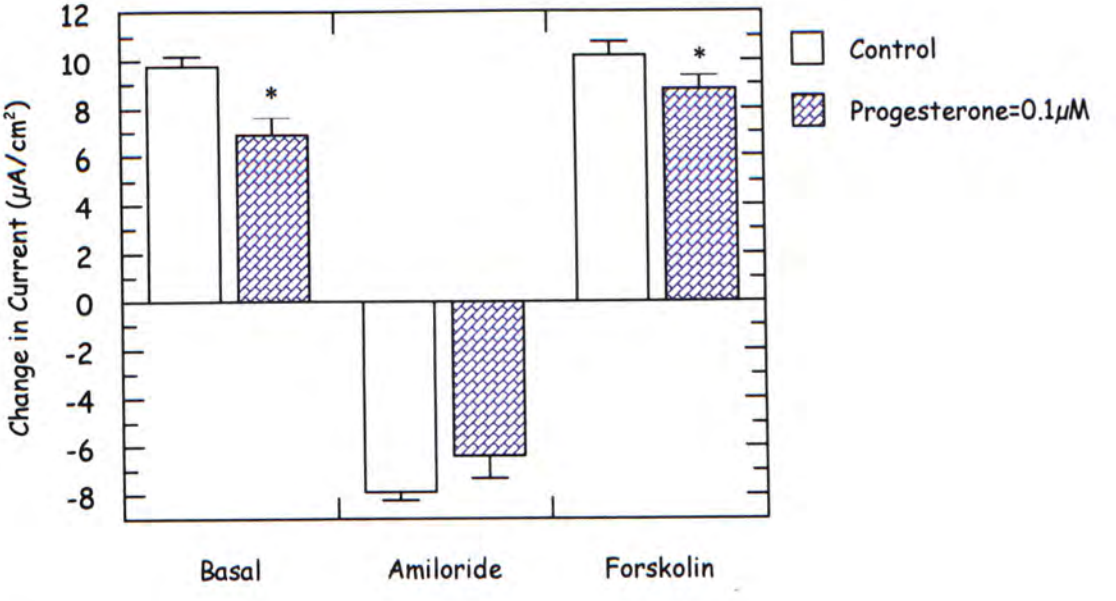


Fig 3.1.9 Effect of 17β-estradiol (0.01 μM) on the forskolin-induced I_{SC} upon 24-hour treatment prior to the experiment. (a) Representative I_{SC} recording showing I_{SC} attributed to amiloride-sensitive basal Na^+ absorption and forskolin-induced Cl^- secretion in control and 0.01 μM 17β-estradiol treatment. (b) Statistical results showing the difference between basal, amiloride-sensitive and forskolin-induced I_{SC} in control and 0.01 μM 17β-estradiol treated epithelium. Data are means ± SEM. (*$P<0.05$, n=6)

(a)



(b)

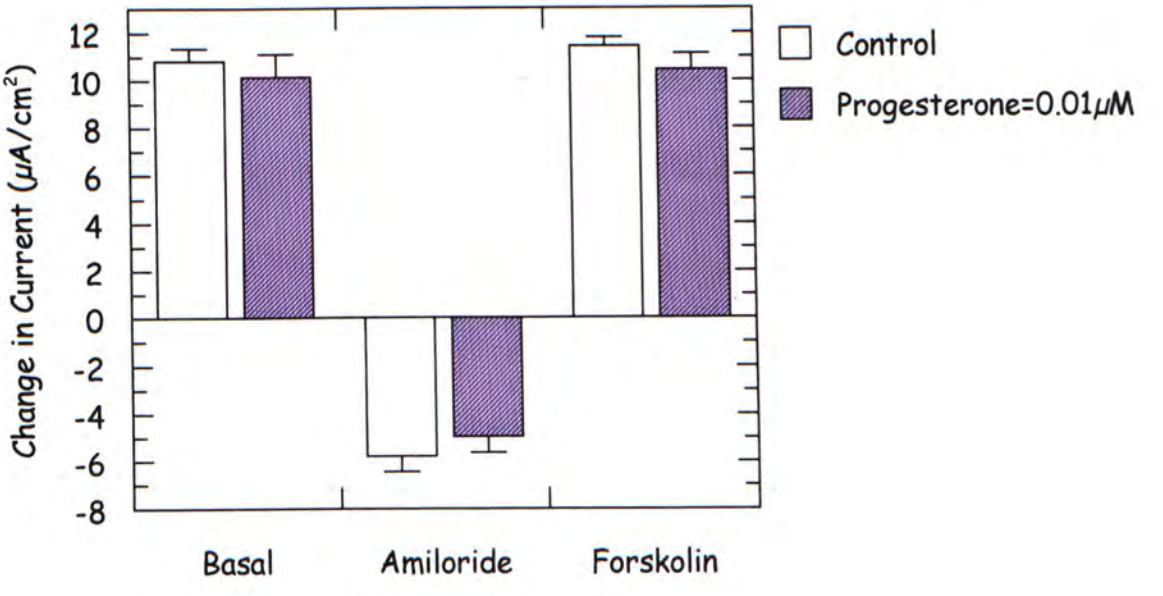


Fig 3.1.10 Effect of progesterone (0.1 μM and 0.01 μM) on the I_{SC} upon 24-hour treatment prior to the experiment. Statistical results showing the difference between basal, amiloride-sensitive and forskolin-induced I_{SC} in (a) control and 0.1 μM progesterone treatment; and in (b) control and 0.01 μM progesterone treatment. Data are means ± SEM. (*$P<0.05$, n=6)

3.2 Culture condition on expression and function of Cystic Fibrosis Transmembrane Conductance Regulator (CFTR) in Mouse Endometrial Epithelial Cells

Summary

Previous studies have demonstrated that cystic fibrosis transmembrane conductance regulator (CFTR), a cAMP-mediated Cl^- channel found in most epithelia including reproductive tract, could be regulated by various culture conditions. The present study further investigated the effect of phenol red, a pH indicator widely used in growth medium, and steroid hormones, present in the supplement fetal bovine serum (FBS), on primary cultured endometrial epithelial cells by monitoring ion channel activities using the short-circuit current technique. When compared to the results obtained with normal medium supplemented with regular FBS, the forskolin-stimulated I_{SC}, presumably mediated by CFTR, obtained in phenol red free medium was significantly reduced, from 16.95 ± 1.53 $\mu A/cm^2$ (control) to 9.72 ± 0.89 $\mu A/cm^2$ (medium without phenol red, $P<0.05$). The forskolin-activated I_{SC} was further attenuated to 5.29 ± 0.46 $\mu A/cm^2$ in the phenol red free medium when supplemented with charcoal/dextran treated FBS where steroid hormones were deprived. Our data suggested that phenol red and steroid hormones present in culture medium and FBS supplement, respectively, may somehow up-regulate CFTR expression *in vitro*. Our study urges a need for carefully choosing the culture media and supplements when the effect of steroid hormones is taken into account.

Introduction

Searching for culture conditions close to the *in vivo* environment have long been carried out in various culture cells. Phenol red, a pH indicator widely used in growth medium, has been found to exhibit minor estrogenic activity, which promotes estroblast proliferation *in vitro* (Ernst et al 1989). In addition, the steroid hormones (estrogen and progesterone) present in the supplement FBS have been shown to modulate cell differentiation in cultured cells (Irwin et al 1991, Kineman et al 1992). However, reports on the effect of phenol red and steroid hormones on ion channel activities are scarce. Previous studies have shown that the expression level of ion channels could be varied by various culture conditions such as culture substrates (Kunzelmann et al 1996, Chan et al 2000b, Chan et al 2000a). The present study further explored the effect of both phenol red and steroid hormones containing FBS on primary cultured mouse endometrial epithelial cells by monitoring ion channel activities using short-circuit technique. The forskolin-stimulated I_{SC}, presumably mediated by CFTR, was attenuated in the medium without phenol red and steroid hormones-removed (charcoal/dextran treated) FBS indicating a role of phenol red and steroid hormones in augmenting CFTR expression *in vitro*.

Results

Effect of phenol red

In order to investigate the effect of phenol red, a pH indicator in growth medium, growth medium without phenol red (phenol-red-free DMEM/F12) was used. As shown in figure 3.2.1, the forskolin-stimulated I_{SC}, presumably the CFTR-mediated Cl^- secretion, was significantly reduced in the medium without phenol-red from 16.95 ± 1.53 μA/cm^2 (control, n=6) to 9.72 ± 0.89 μA/cm^2 (phenol-red-free medium, n=6, $P<0.01$).

Effect of steroid hormones

To further explore the effect of steroid hormone(s), present in FBS, charcoal/dextran treated FBS (steroid hormones-removed FBS) was studied. In medium without phenol red and steroid hormones-removed FBS, the forskolin-activated I_{SC} was substantially reduced to 5.29 ± 0.46 μA/cm^2 ($P<0.01$, Fig. 3.2.1). In addition, the forskolin-sensitive I_{SC} was sensitive to DPC, a Cl^- channel blocker knows to inhibit CFTR, and the DPC-sensitive I_{SC} was significantly reduced from 13.14 ± 1.23 μA/cm^2 (control) to 6.37 ± 0.48 μA/cm^2 (phenol red free medium, $P<0.01$). Our data suggest that the steroid hormone(s) (estrogen and progesterone) in the certified FBS together with phenol red play a role in increasing CFTR-mediated I_{SC}. Note that no significant difference in amiloride-sensitive I_{SC} was observed between the control medium and phenol red or steroid hormones-removed media.

Discussion

Uterus is one of the organs expressing CFTR. Recently, we have established a primary culture of mouse endometrial epithelial cells which exhibit basal amiloride-sensitive current, presumably mediated by epithelial Na^+ channels (ENaC), and CFTR activity upon stimulation. Our previous studies on the cultured endometrial cells have demonstrated the involvement of CFTR in mediating a number of neurohormonal responses (Chan et al 1997b, Chan et al 1997a, Chan et al 1999, Fong & Chan 1998). However, the details as to how culture conditions may affect CFTR expression have not been elucidated. The present study has demonstrated a role of phenol red and steroid hormones in augmenting the CFTR-mediated Cl^- secretion in cultured mouse endometrial epithelial cells.

CFTR expression has been reported to be influenced by ovarian hormones. Rochwerger et al (Rochwerger & Buchwald 1993, Rochwerger et al 1994) have demonstrated the up-regulation of CFTR expression both *in vivo* and *in vitro*. On the other hand, down-regulation of CFTR expression by progesterone has been shown in endometrial epithelial cells using semi-quantitative RT-PCR (Mularoni et al 1995). Phenol red, a pH indicator used in most culture medium, is a weak estrogen which can stimulate some estrogen-sensitive cells (Welshons et al 1988). Being structurally similar to the ovarian hormone, estrogen, phenol red could work through the same pathway as estrogen by binding to the estrogen receptor and modulate CFTR expression. Taken together, our present study showing a substantial reduction in the CFTR-mediated I_{SC} in phenol red-free medium and steroid hormones removed FBS

was consistent with the dependency of CFTR expression on estrogen. However, the actual mechanisms and pathways that phenol red and steroid hormones do to increase the CFTR-mediated Cl^- secretion remain to be elucidated.

Interestingly, the amiloride-sensitive basal I_{SC}, presumably mediated by ENaC, did not vary much in the same preparation suggesting that these culture agents may not act the same way as ovarian hormones do.

In conclusion, the fact that CFTR activities are affected by culture condition such as steroid hormones in FBS and phenol red raises a need for better defined culture conditions when accessing the hormonal effects on ion channels *in vitro*.

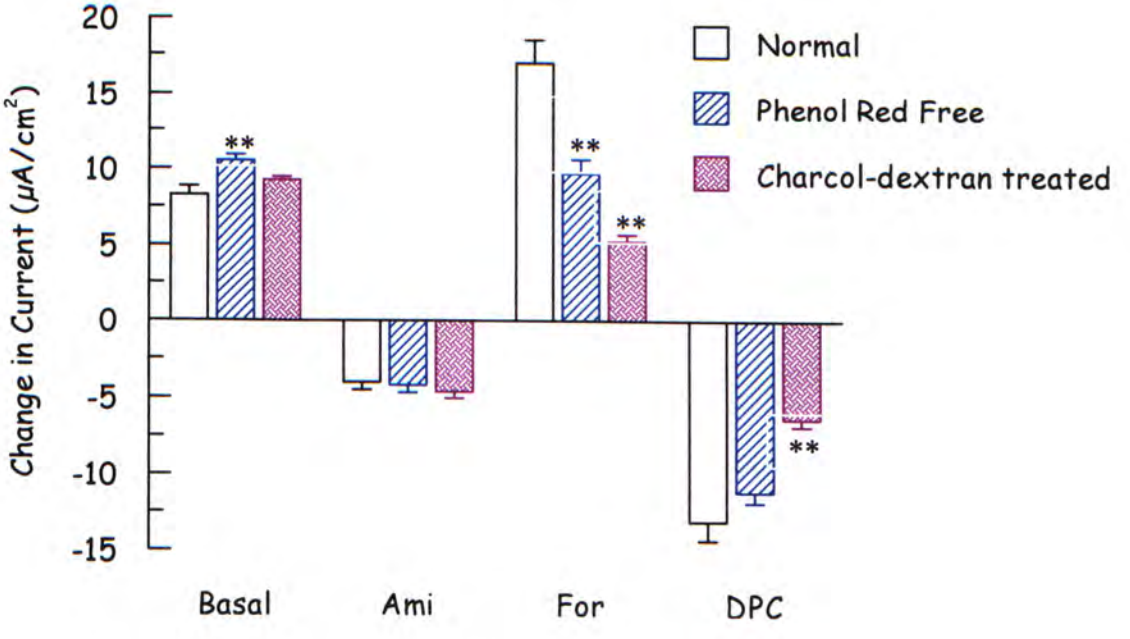


Fig. 3.2.1 Summary of the effect of different culture media on I_{SC}. The four groups show the current magnitude of the basal, amiloride-sensitive, forskolin-stimulated and DPC-blockable I_{SC}. Data are means ± SEM. (**P<0.01, n=6)

3.3 Expression regulation of endometrial epithelial Na^+ channel (ENaC) subunits and cystic fibrosis transmembrane conductance regulator (CFTR) by Na^+ diet during the estrus cycle in mice

Summary

Previous studies have demonstrated the cyclic changes in regulating ENaC and CFTR expression by ovarian hormones during the estrus cycle. The present study investigated the regulation of ENaC subunits and CFTR expression by aldosterone *in vivo* using a low Na^+ diet mouse model. Estrus cycle of 10-12 weeks old ICR mice was synchronized by injection of estrogen (5 μg/100g) two days before experiment. The mice were fed with a low sodium diet for at least two weeks, during which circulating aldosterone is expected to be elevated. The mRNA level of uterine CFTR and ENaC subunits for all stages of the estrus cycle was examined by competitive reverse transcription-polymerase chain reaction (RT-PCR). The results showed a cyclic expression pattern of uterine ENaC throughout the estrus cycle with maximal expression level at the metestrus for β subunit and at diestrus for α and γ subunits. On the other hand, the expression pattern of CFTR with maximal expression level at proestrus and estrus and decrease at metestrus and diestrus. When fed with a low Na^+ diet, which was expected to elevate the circulating aldosterone, a steroid hormone which controls the rate of transepithelial Na^+ absorption in a variety of epithelia, the cyclic characteristics of expression pattern for all ENaC subunits remained unchanged but for CFTR was changed, it shows lower mRNA expression in all stage of estrus cycle. And the maximal

level of ENaC expression was considerably elevated, 121.9 %, 35 % and 283.3 % for α, β and γ subunit, respectively. The results indicate that when undergoing a low Na+ diet, uterine ENaC expression was maximally elevated, presumably in response to elevated circulating aldosterone.

The present study also investigated the functional expression of ENaC and CFTR upon aldosterone treatments *in vitro* using short-circuit current (I_{SC}) technique. The amiloride-sensitive I_{SC} was increased from 7.67 ± 3.62 $\mu A/cm^2$ to 19.54 ± 1.05 $\mu A/cm^2$ (aldosterone, $n=14$, $P<0.01$) indicating the upregulation of ENaC by this steroid hormone. This suggests that in addition to the regulation by ovarian hormones, ENaC expression level also responds to changes in circulating aldosterone, contributing to the overall body electrolyte and fluid homeostasis. Our data also indicated a predominant role of γENaC subunit in regulating ENaC activity and thus the rate of Na^+ absorption across mouse endometrium.

Introduction

The amiloride-sensitive epithelial Na^+ channel (ENaC) controls the rate of transepithelial Na^+ absorption in a variety of epithelia including the kidney, colon, airways and secretory ducts of several glands (Garty & Palmer 1997, Horisberger 1998). Aldosterone is a steroid hormone which enhances the Na^+ reabsorption in various organs. In kidney, aldosterone is produced by the glomerulosa cells of the adrenal cortex and has a number of important actions on the kidneys. Aldosterone stimulates Na^+ reabsorption by the principal cells in the late portion of the distal tubule and collecting duct. Aldosterone enters the cell and binds to a cytoplasmic receptor. The hormone-receptor complex interacts with specific binding sties on the DNA and thereby regulates the transcription of mRNA. Translations of these messages increase the levels of a number of proteins important in the process of Na^+ reabsorption by the cell. These aldosterone-induced proteins may include new ENaC in apical membrane and Na^+-K^+-ATPase pump at basolateral membrane. By these actions Na^+ entry into the cell across the apical membrane is enhanced. Thus aldosterone increases the reabsorption of NaCl from the tubular fluid. ENaC is made of three different but homologous subunits (α, β, and γ), each of which is believed to play a role in regulating ENaC activity (Cannessa et al 1993, Canessa et al 1993, McDonald et al 1995, McDonald et al 1994). The importance of these individual subunits can be highlighted by human diseases such as Liddle syndrome or pseudohypoaldosteronism which are caused by mutations in individual ENaC subunits resulting in gain or loss of ENaC function (Chang et al 1996, Hummler & Horisberger 1999). However, little is known about aldosterone effect on

CFTR.

Previous studies have demonstrated a functional role of both ENaC and CFTR in mediating the absorptive and secretory activities of the endometrium, respectively. We have also found variation in the expression patterns of uterine CFTR and ENaC with estrous cycle indicating a tight coupling between ovarian hormonal levels and ion channel expression. However, what happens to uterine ENaC and CFTR expression when the state of Na^+ homeostasis is disturbed, such as in a low Na diet? Can these channel's expression level respond to elevated circulating aldosterone? In other words, do ENaC and CFTR contribute to overall body electrolyte and fluid balance? In order to study a possible regulatory role of endogenous aldosterone in endometrial ion channels, the present study examined the expression pattern of uterine CFTR and ENaC mRNA level throughout the estrus cycle of mice fed with a low Na diet. The effect of aldosterone on functional expression of ENaC and CFTR in cultured endometrial cells was also examined using the short-circuit current technique.

Result

Effect of low Na diet on uterine ENaC and CFTR expression

To study how uterine ENaC and CFTR expression response to low Na^+-intake, female adult ICR mice aged 10-12 weeks were fed with a low sodium diet for at least two weeks. Their estrus cycles were synchronized by injection of estrogen (5 μg/100g) intraperitoneally two days before experiment. The stage of estrus cycle was identified by examining the morphological changes of the vagina.

The expression of ENaC and CFTR at different stages of estrus cycle was demonstrated by semi-quantitative RT-PCR with GAPDH served as an internal marker. All ENaC subunits (α-, β-, and γ-) mRNA expression exhibited similar pattern throughout the estrus cycle. The upper bands of agarose gels shown in figure 3.3.1a, 3.3.2a and 3.3.3a represent α- (790 bp), β- (420 bp) and γ-ENaC (724 bp), respectively. α- and γ-ENaC mRNA expression level was low at proestrus and estrus and gradually increased at metestrus and reached at maximum at diestrus in mice fed with normal and low Na diet (Fig 3.3.1a and Fig 3.3.3a). Note that the maximal mRNA expression level of α and γENaC was considerably elevated in low Na diet, 121.9 % and 283.3 %, respectively. β-ENaC expression pattern was not altered throughout the estrus cycle (Fig 3.3.2a). A relative intensity ratio of α-, β- and γ-ENaC to GAPDH was plotted against different estrus stage and shown in figure 3.3.1b, 3.3.2b and 3.3.3b respectively.

Figure 3.3.4 shows the uterine CFTR mRNA expression during estrus cycle of control and mice on low sodium diet. As shown in figure 3.3.4a, the upper band is

CFTR (481 bp) and the low band is GAPDH (340 bp), which act as an internal marker for each sample. In normal diet, CFTR mRNA expression is considerably high at proestrus and estrus and low at metestrus and diestrus (Fig 3.3.4a). When fed with a low Na^+ diet, the CFTR expression pattern was altered; mRNA expression level was low throughout estrus cycle. The data indicate the differential expression of uterine ENaC and CFTR in mice fed on low Na^+ diet, presumably due to the elevated circulating aldosterone.

Effect of aldosterone on ENaC and CFTR-mediated I_{SC}

To explore the effect of aldosterone *in vitro*, 1 μM of aldosterone was added to the medium and the endometrial cells 24 hours prior to the experiment. As shown in figure 3.3.5, the basal I_{SC} of aldosterone-treated epithelia was significantly increased from 11.37 ± 1.79 μA/cm^2 (control, n=6) to 18.90 ± 0.83 μA/cm^2 (aldosterone, $P<0.05$, n=14). The amiloride-sensitive I_{SC} was significantly increased from 7.67 ± 3.62 μA/cm^2 to 19.54 ± 1.05 μA/cm^2 ($P<0.05$). The forskolin-induced I_{SC} was increased from 3.08 ± 1.16 μA/cm^2 to 5.97 ± 0.74 μA/cm^2 ($P<0.05$) in aldosterone treated epithelia. The present results indicate that the functional expression of both CFTR and ENaC in the endometrium could be regulated by aldosterone *in vitro*.

Discussion

The present study is the first to demonstrate the contribution of uterine ENaC and CFTR to Na^+ homeostasis. Elevation of circulating aldosterone level, which results in the upregulation of renal ENaC activity, is how our body responds to insufficient Na intake (Aguilera & Catt 1978). Our previous studies have demonstrated substantial amiloride-sensitive Na^+ absorption, mediated by epithelial sodium channel (ENaC) under unstimulated condition and CFTR-mediated Cl^- secretion upon neurohormonal stimulation in primary cultured mouse endometrial epithelial cells (Chan et al 1997c, Chan et al 2000a, Chan et al 1999). However, how these uterine ion channels respond to low Na intake has not been reported.

The cyclic expression pattern of all ENaC subunit mRNA remained unchanged in low Na diet group. However, α and γENaC mRNA levels were considerably increased at diestrus in mice fed with low Na diet as compared to one fed with normal diet. This increase in α and γ ENaC expression level is probably due to the elevation of aldosterone level in low Na diet treatment. The supporting evidence came from the significant increment in the amiloride-sensitive I_{SC} in aldosterone-treated endometrial monolayers. Differential expression of different ENaC subunits in response to aldosterone has been observed in different tissues. Aldosterone caused a marked increase in α-ENaC mRNA but minor changes for β- and γ-ENaC in mouse kidney (MacDonald et al 2000). Elevated circulating aldosterone, due to either dietary NaCl restriction or aldosterone infusion, markedly increased the amount of α-ENaC protein without increasing the amount of β- and γ-ENaC subunits in both mouse (Loffing et al

2000) and rat (Masilamani et al 1999) kidney. However, β- and γ-ENaC mRNA expression was enhanced in rat distal colon by endogenous aldosterone stimulation (Lingueglia et al 1994, Renard et al 1995). A recent study examining both ENaC activity and mRNA expression in identical rat distal colon has also confirmed that aldosterone effect on ENaC activity is regulated by aldosterone-dependent transcriptional control of its β- and γ-subunits (Epple et al 2000). In cultured airway epithelial cells, treatment with aldosterone resulted in enhanced α-ENaC mRNA expression (β- and γ-subunits were not examined) and increased Na^+ conductance (Epple et al 2000, Kunzelmann et al 1996). Our recent studies have demonstrated the up-regulation of ENaC activity associated with an increase in γ-ENaC subunit by changing the culture conditions, Matrigel coating as well as aldosterone treatment, suggesting an important role of γ-ENaC in determining uterine ENaC activity (see next section). The present study provided physiological evidence showing the substantial increase of α and γ-ENaC in the endometrium of mice fed with low Na diet at diestrus. It should be noted that the C-terminal of γ-ENaC subunit has a motif known to interact with a ubiquitin protein-ligase, mutations of which cause Liddle syndrome (Firsov et al 1996, Snyder et al 1995, Snyder 2000). The differential regulation of aldosterone on different tissue could be due to tissue-specific transcription efficiency.

In contrast to unchanged cyclic ENaC expression pattern, uterine CFTR mRNA expression was altered in low Na diet. CFTR mRNA was maximally detected at proestrus and estrus in normal diet group, but was entirely suppressed and remained

constantly low throughout the estrus cycle. The down-regulation of CFTR expression at proestrus and estrus in low Na diet group suggested involvement of CFTR in Na^+ homeostasis in addition to ENaC. Differential regulation of Cl^- transport by aldosterone has also been observed in other tissues. Studies on mouse cortical collecting duct (mCCD) cells have demonstrated aldosterone increased net Cl^- reabsorption (Duong et al 1998). Two Cl^- channels of conductance 46 pS and 9 pS have been identified in CCD cells (Ling et al 1994, Sansom et al 1990), in which the 9pS Cl^- channel can be activated by PGE_2, forskolin and cAMP. However, nothing is known about which Cl^- channel(s) mediated the aldosterone-elicited Cl^- transport in mCCD cells. Nevertheless, enhancement of Cl^- secretion, rather than Cl^- absorption, by low sodium condition and aldosterone has also been demonstrated in hen colon (Clauss et al 1988) putting forward an entirely opposite role of aldosterone in regulating Cl^- transport.

The present study has demonstrated the role of aldosterone in regulating uterine ion channels activity both *in vivo* and *in vitro*. However, it should be noted that there is a discrepancy between the *in vivo* and *in vitro* effect of aldosterone on CFTR. CFTR expression level was suppressed at proestrus and estrus in low Na diet. However, CFTR activity was increased in endometrial epithelia treated with aldosterone in the short-circuit current experiment. This discrepancy may be due to the fact that there are more complicated factors such as ovarian hormones, other than aldosterone, involved in co-regulating CFTR *in vivo*, the detail of which awaits investigation.

In normal estrus, all ENaC subunits expression level was low at proestrus and

estrus and reached a maximal level at either metestrus or diestrus. This expression pattern favors the reduction of uterine fluid volume in the lumen during the later stage of the cycle to enhance close contact between the endometrial surfaces to facilitate the implantation of embryo. Interestingly, the expression pattern of all uterine ENaC subunits mRNA remained unaltered under a low Na diet. However, the maximal expression levels of α and γ ENaC subunits, but not β ENaC, were significantly elevated in response to the low Na diet, presumably due to the elevation of circulating aldosterone. Our data indicate an important role of α and γ ENaC subunits in determining uterine ENaC activity and their contribution to Na^+ homeostasis.

Uterine CFTR mRNA expression level exhibits a maximal level at proestrus and estrus, and was reduced to a minimal level at metestrus and diestrus. However, uterine CFTR mRNA level was suppressed and remained low throughout the cycle in a low Na diet. The importance of CFTR in the uterus could be emphasized by the convergence of a number of neurohormonal agents on it in cultured endometrial cells (Chan et al 1999). Activation of CFTR, which is a cAMP-activated chloride channel, will drive chloride ion and its counter ion sodium from the blood to the lumen. Maximal expression of CFTR at proestrus and estrus is important in uterine fluid formation, which may be critical for sperm capacitation and the sperm transit through the uterus. Suppression of uterine CFTR expression under a low Na diet will result in less NaCl and fluid secretion which may disrupt some reproductive events, but nevertheless, is an alternative way to preserve Na and body fluid when Na^+ homeostasis is disturbed. The present study suggested a role of uterine CFTR in Na^+

homeostasis in addition to its important role in reproductive events.

Taken together, altered cyclic expression pattern of CFTR and the elevation of α- and γ-ENaC expression in low Na diet in the present study suggest that Na^+ homeostasis is important in determining both ENaC and CFTR expression indicating that these ion channels are involved in overall body function. Our data also indicated that uterus is also a site for regulation of electrolyte and fluid in homeostasis. The detail mechanisms that Na^+ depletion affects uterine ENaC and CFTR expression await further elucidation.

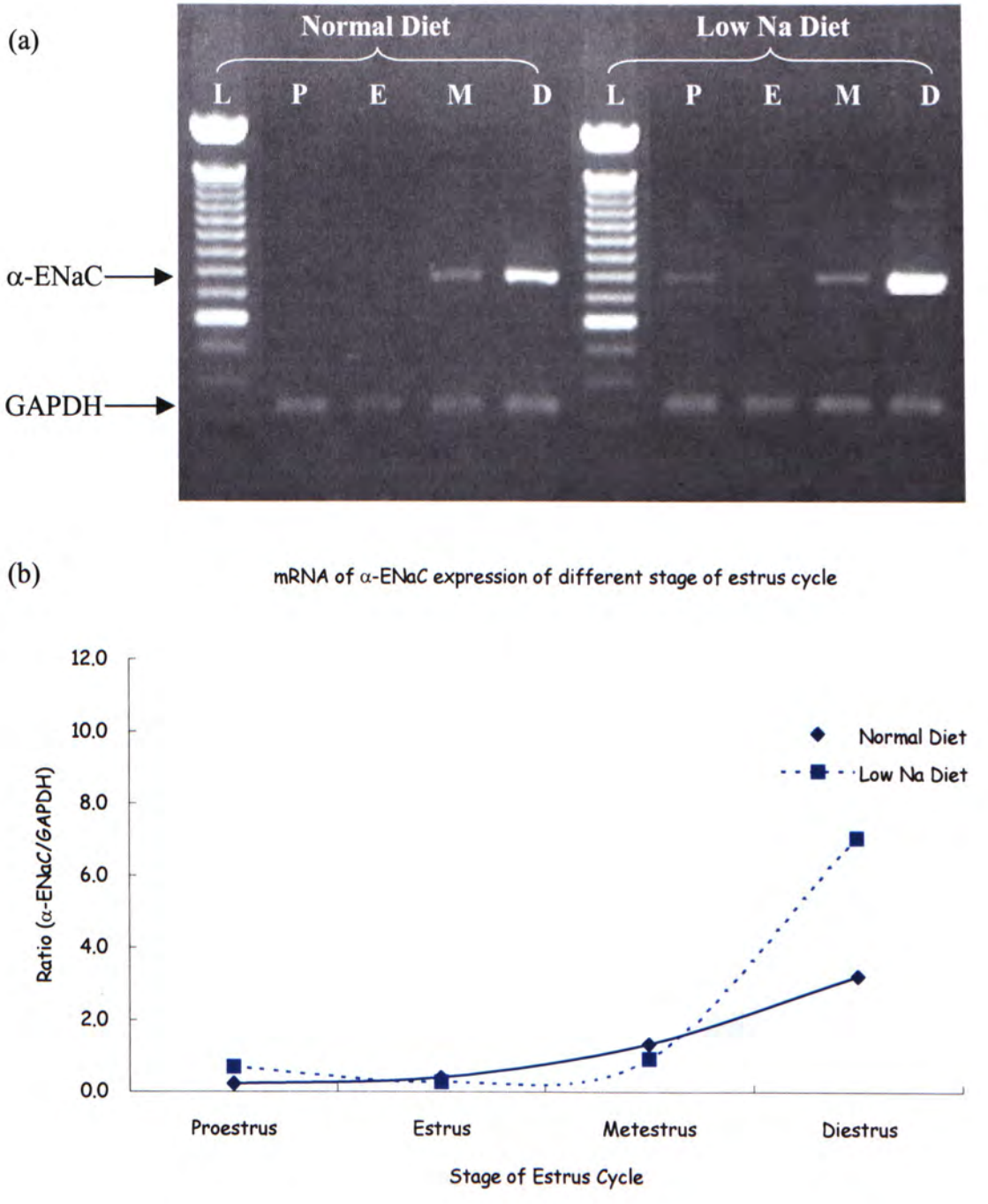
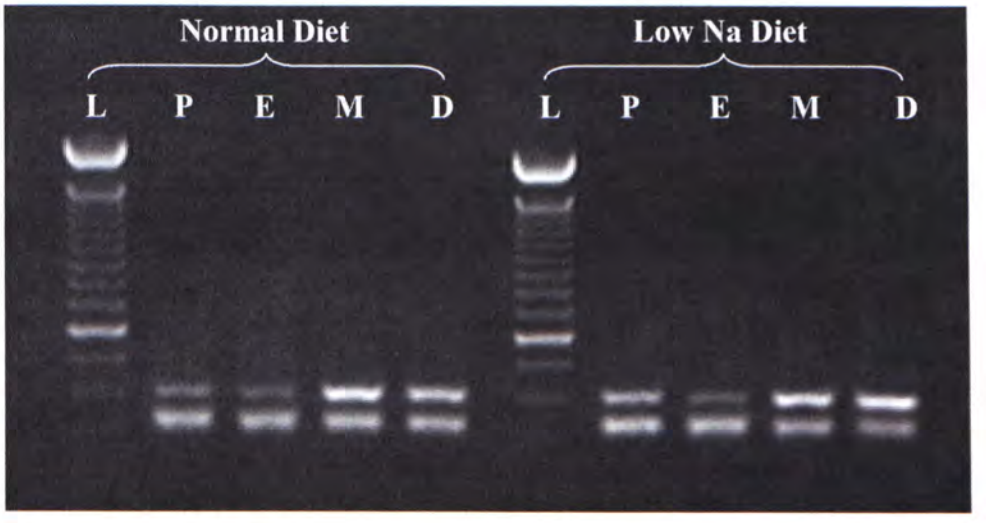


Fig 3.3.1 Effect of low Na diet on uterine α–ENaC mRNA expression. (a) agarose gel showing different expression level of α-ENaC (790 bp) and GAPDH (340 bp) at proestrus (P), estrus (E), metestrus (M) and diestrus (D). 'L' stands for 100 bp DNA ladder. GAPDH was used as an internal marker. (b) Intensity ratio of α-ENaC to GAPDH at different stage of the estrus cycle.

(a)



(b)

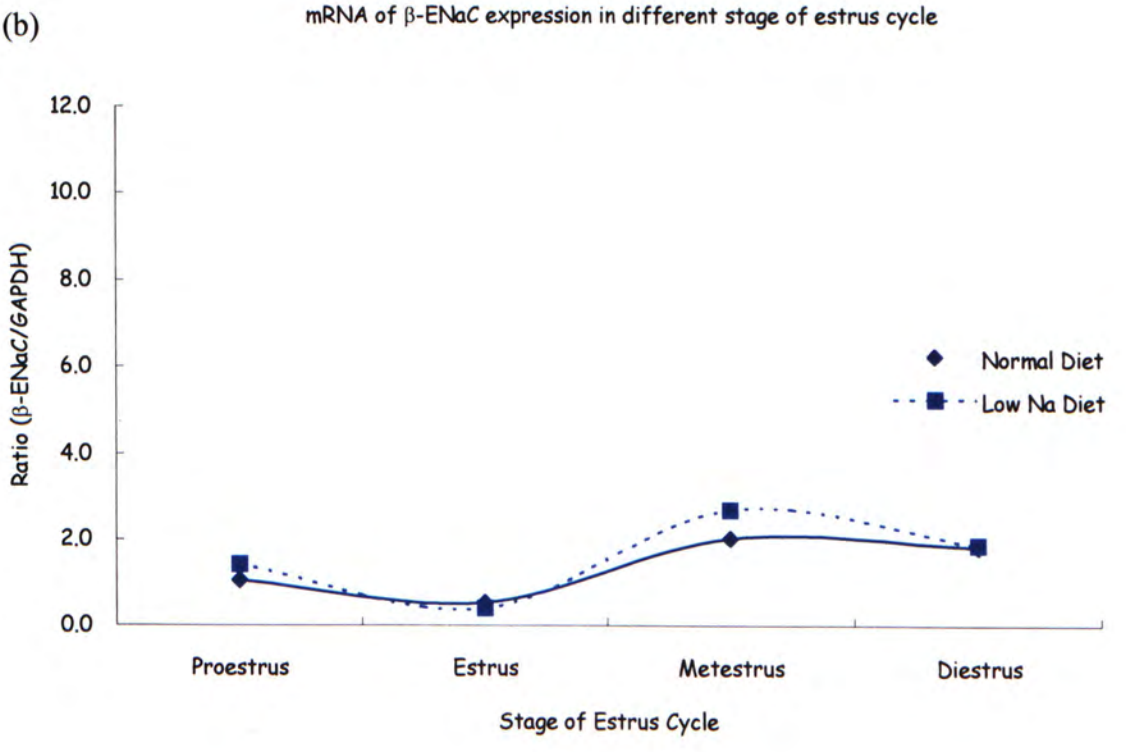
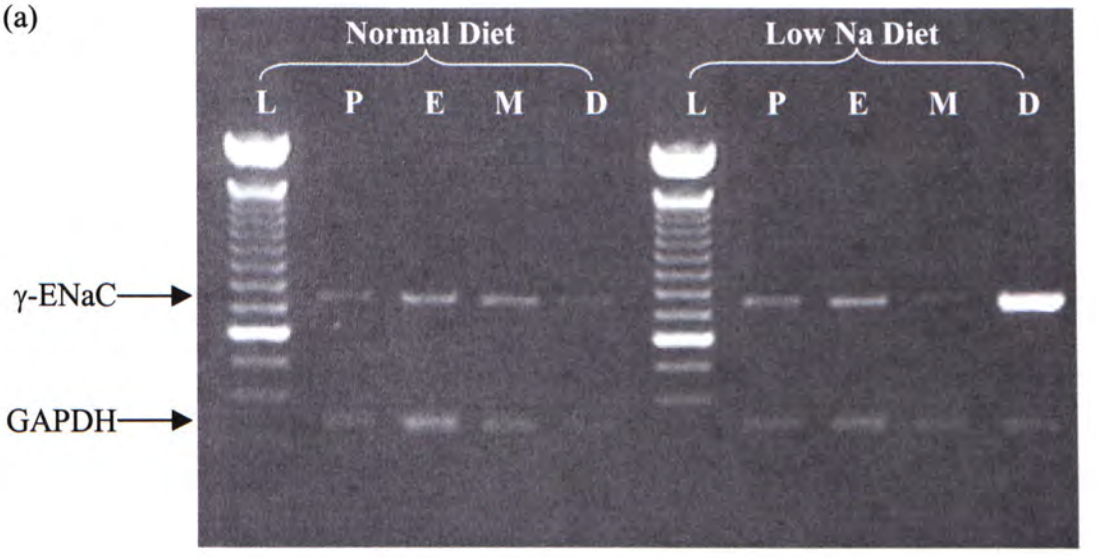


Fig 3.3.2 Effect of low Na diet on uterine β–ENaC mRNA expression. (a) agarose gel showing different expression level of β-ENaC (420 bp) and GAPDH (340 bp) at proestrus (P), estrus (E), metestrus (M) and diestrus (D). (b) Intensity ratio of β-ENaC to GAPDH at different stage of estrus cycle.

(a)



(b)

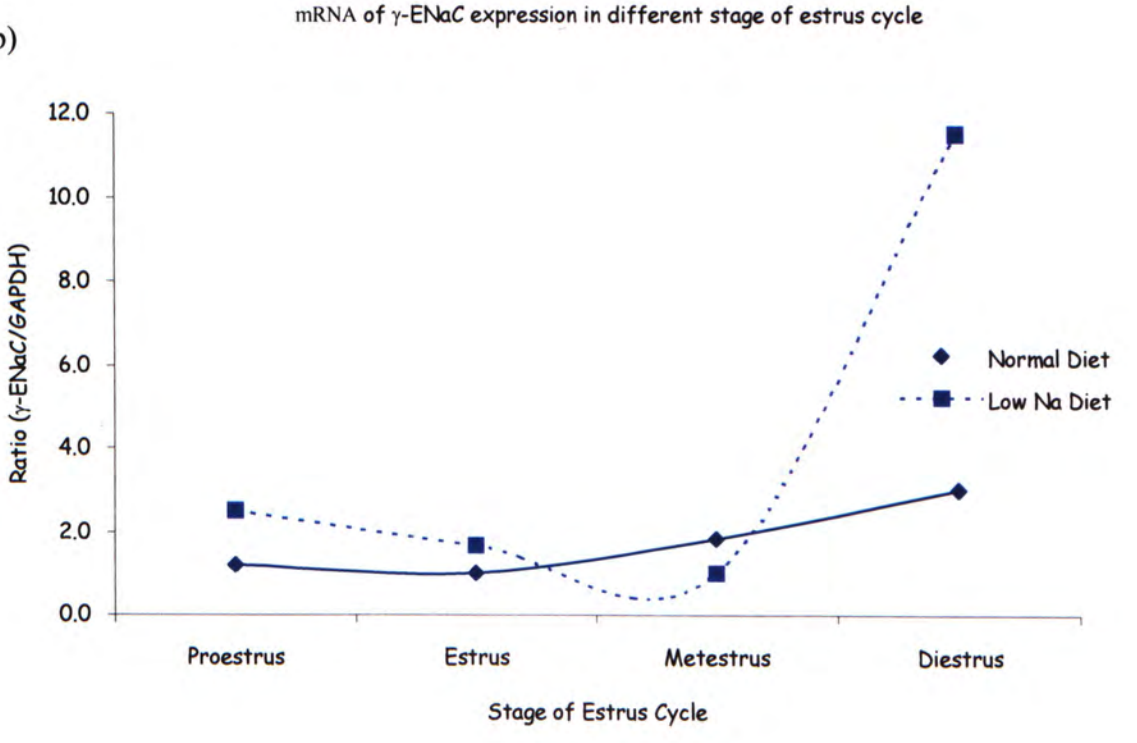


Fig 3.3.3 Effect of low Na diet on uterine γ–ENaC mRNA expression. (a) agarose gel showing different expression level of γ-ENaC (720 bp) and GAPDH (340 bp) at proestrus (P), estrus (E), metestrus (M) and diestrus (D). (b) Intensity ratio of γ-ENaC to GAPDH at different stage of estrus cycle.

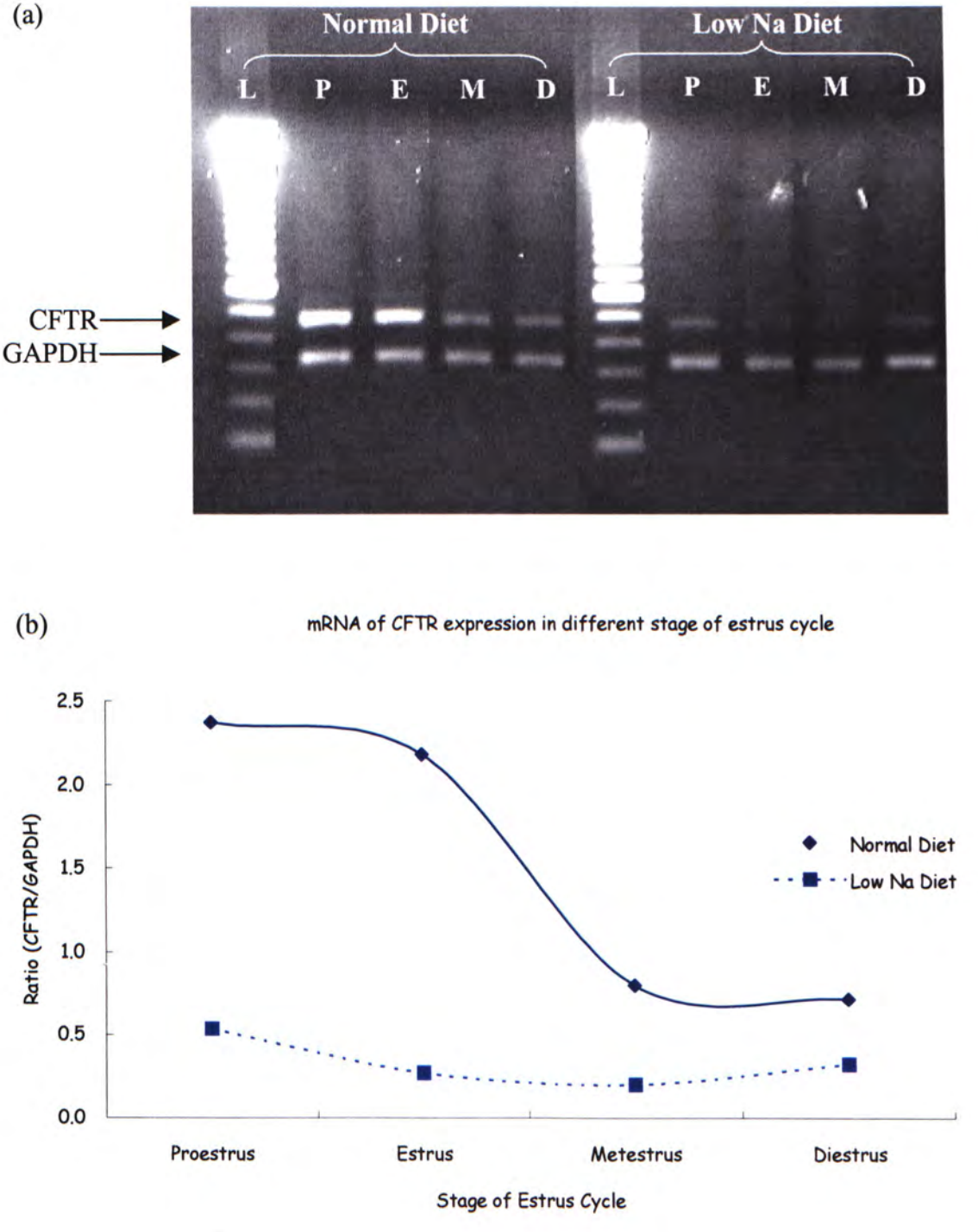


Fig 3.3.4 Effect of low Na diet on uterine CFTR mRNA expression. (a) agarose gel showing different expression of CFTR (481 bp) and GAPDH (340 bp) at proestrus (P), estrus (E), metestrus (M) and diestrus (D). (b) Intensity ratio of CFTR to GAPDH at different stage of estrus cycle.

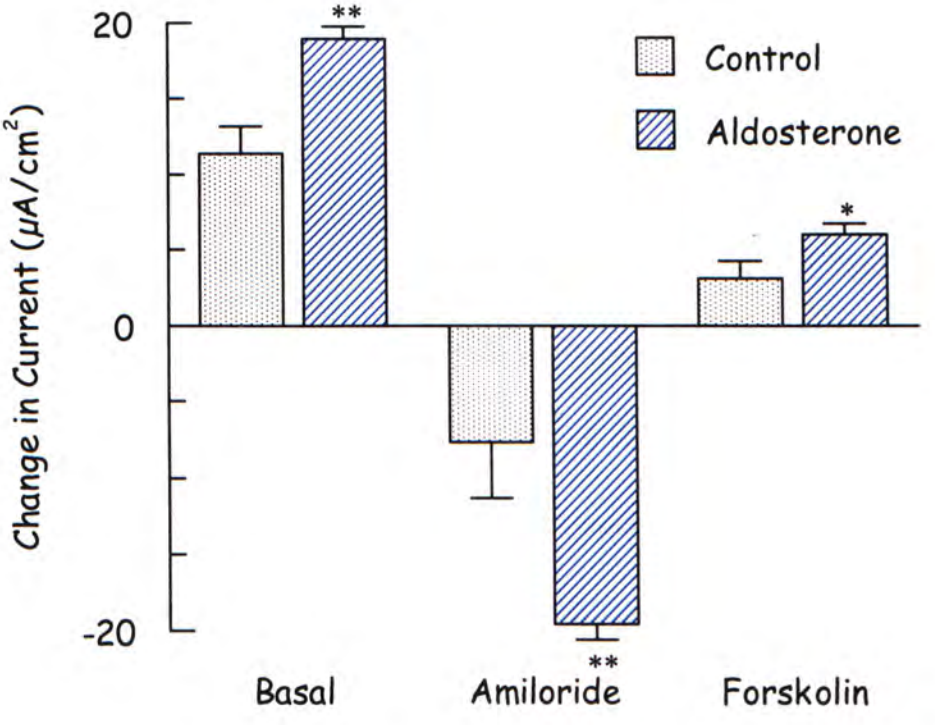


Fig 3.3.5 Effect of aldosterone on I_{SC}. The current changes of basal, amiloride-sensitive and forskolin-induced I_{SC} upon aldosterone 1 μM (n=14) treatment 24 hours prior to the experiment. Data are means ± SEM. (*P<0.05 and **P<0.01)

3.4 Enhanced epithelial Na^+ channel (ENaC) activity in mouse endometrial epithelium by upregulation of γENaC subunit

Summary

The amiloride-sensitive epithelial Na^+ channel (ENaC), which is made of three different but homologous subunits, controls the rate of transepithelial Na^+ absorption in a variety of epithelia. The present study investigated the functional role of its subunits in regulating ENaC activity, measured as amiloride sensitive short-circuit current (I_{SC}), in the mouse endometrial epithelium under different culture conditions. Treatment of the cultured epithelia with aldosterone (1 μM) or culturing cells on filters coated with concentrated Matrigel resulted in an increase in the amiloride-sensitive I_{SC}. Semi-quantitative RT-PCR demonstrated that the expression of α and β subunits was not significantly altered by these treatments, but an increase in γ subunit expression was observed. An 11-fold increase, induced by aldosterone, in the expression of γ subunit, but not α and β subunits, was confirmed by capillary electrophoresis with laser-induced fluorescence (CE-LIF). Treatment of endometrial cells with antisense against γENaC subunit abolished the aldosterone-enhanced amiloride-sensitive I_{SC}. The results indicated an important role of γENaC subunit in determining ENaC activity and a possible role of γENaC subunit in interacting with CFTR was also discussed.

Introduction

The amiloride-sensitive epithelial Na^+ channel (ENaC) controls the rate of transepithelial Na^+ absorption in a variety of epithelia including the kidney, colon, airways and secretory ducts of several glands (Garty & Palmer 1997, Horisberger 1998). ENaC is made of three different but homologous subunits (α, β, and γ), each of which is believed to play a role in regulating ENaC activity (Cannessa et al 1993, Canessa et al 1993, McDonald et al 1994, Hopfer et al 1994). The importance of these individual subunits can be highlighted by human diseases such as Liddle's syndrome or pseudohypoaldosteronism which are caused by mutations in individual ENaC subunits resulting in gain or loss of ENaC function (Chang et al 1996, Hummler & Horisberger 1999). However, the precise role of each ENaC subunit in regulating ENaC activity is far from understood; particularly, no evidence has been provided linking ENaC activity directly to specific ENaC subunits in tissues where they are normally expressed. In the present study, ENaC activity in the mouse endometrial epithelium, measured as amiloride sensitive short-circuit current (I_{SC}), was upregulated by changing culture conditions. The enhanced ENaC activity was correlated with an increase in the mRNA expression of γ subunit and abolished by antisense against γENaC.

Results

Increased ENaC activity by culture conditions

Previous studies have shown that cultured endometrial epithelia exhibit a basal I_{SC} which can be substantially blocked by amiloride, indicative of predominant Na^+ absorption (Chan et al 1997b, Chan et al 1997a, Chan et al 1999). Recently, we have found that the ENaC activity of endometrial cultures could be affected by coating the permeable support with Matrigel (Chan et al 2000b), which resembles the extracellular matrix facilitating rapid epithelial reconstitution and differentiation (Lacy 1995, Rawdon 1998). As shown in Fig 3.4.1a, the amiloride-sensitive I_{SC} obtained from endometrial cells cultured on double-Matrigel-coated filters (n=15) was significantly increased ($P<0.001$) by more than 8 folds as compared to that obtained from non-Matrigel-treated cultures (n=27). When the cultures were treated with Matrigel (single-coated, n=3), further increase ($P<0.05$) in the amiloride-sensitive I_{SC} was observed when aldosterone (1 μM, n=10) was added to the culture medium (Fig 3.4.1b). These results suggest that ENaC activity could be regulated by different mechanisms, either by factors such as Matrigel affecting cellular differentiation or hormones such as aldosterone.

Differential expression of ENaC subunits

The expression of ENaC subunits in non-Matrigel-treated and double-matrigel-coated cultures or aldosterone-treated cultures was examined by semi-quantitative RT-PCR using primers designed from mouse sequences for ENaC subunits (α: 2171 – 2960 bp; β: 1961 – 2380 bp; γ: 2070 –2793 bp) (Ahn et al 1999). The intensities of the bands of ENaC subunits were normalized to that of GAPDH (Sabath et

al 1990) which was amplified simultaneously. RT-PCR products were further measured by CE-LIF technique described previously (Fiscus et al 2001). All experiments were repeated 3 times and similar results were obtained. As shown in Fig 3.4.2a, bands at 790, 420 and 724 bp as expected for ENaC α, β and γ subunit, respectively, were obtained. The expression of both α and β subunits was not significantly altered with treatment of either Matrigel or aldosterone. However, prominent change in γENaC expression was observed. In cultures without treatment of Matrigel, the expression of γENaC was hardly detectable in agarose gel (Fig 3.4.2.a), corresponding to a minimal amiloride sensitive I_{SC} (Fig 3.4.1a). Much enhanced expression of γENaC was observed with double-Matrigel cultures (Fig 3.4.2a), corresponding to a 8-fold increase in ENaC activity (Fig 3.4.1a). Single-Matrigel-treated cultures gave rise to a detectable level of γENaC which was further enhanced by treatment with aldosterone (Fig. 3.4.2b). Quantitative measurement of mRNAs of ENaC subunits was further carried out using CE-LIF which has been shown to be a sensitive method of detection and quantitation of PCR products (Personett et al 1997). As demonstrated in Fig. 3.4.3, CE-LIF revealed that the PCR product level of αENaC and βENaC was not altered by aldosterone, but an 11-fold increase was observed with γENaC. The correlation between the expression level of γENaC and the measured ENaC activity indicates that the effect of Matrigel and aldosterone on ENaC activity may be mediated by transcriptional regulation of γENaC subunit.

Effect of Antisense against γENaC

To further test whether γENaC subunit was indeed responsible for enhancing ENaC activity, experiments were conducted using antisense against γENaC subunit. As shown

in Fig.3.4.4, the aldosterone-enhanced amiloride-sensitive I_{SC} was abolished by γENaC antisense ($n=6$, $P<0.05$), but not by the mis-sense (control, $n=6$, $P>0.05$), confirming that γENaC subunit was indeed responsible for the enhancement in ENaC activity induced by aldosterone.

Discussion

Aldosterone is the key hormone in the regulation of Na^+ homeostasis. Its Na^+-saving action is mediated by the activity of ENaC located in the apical membrane aldosterone-responsive tissues such as the kidney and distal colon. Differential expression of different ENaC subunits in response to aldosterone has been observed in different tissues with marked increase in αENaC mRNA in mouse and rat kidney (MacDonald et al 2000, Loffing et al 2000, Masilamani et al 1999) but enhanced β and γENaC mRNA expression in rat distal colon (Renard et al 1995, Lingueglia et al 1994, Epple et al 2000). These differences could be due to tissue-specific transcription efficiency, and, no firm conclusion can be drawn as to which specific subunit is responsible for aldosterone-enhanced ENaC activity since no direct demonstration has been made linking transcriptional changes directly to functional variations in ENaC activity.

The present study observed an increase in the amiloride-sensitive I_{SC} associated with an increase in expression of γENaC, but not α and β subunits, in response to aldosterone stimulation. The enhancing effect of aldosterone on ENaC activity was reversed by antisense against γENaC, demonstrating directly for the first time that the change of γENaC at transcriptional level was indeed responsible for the enhancement of ENaC activity induced by aldosterone. Another interesting observation made in the present study was that Matrigel, which resembles the extracellular matrix facilitating

epithelial differentiation, also enhanced endometrial ENaC activity with an increase in γENaC expression. While the effect of aldosterone may be mediated by well known nuclear receptors, it is not clear the exact mechanism by which Matrigel may act through an extracellular mechanism although it cannot be ruled out that the growth factors contained in the Matrigel may be responsible for the enhancement of ENaC expression. Nevertheless, the upregulated ENaC activity in both cases, aldosterone and Matrigel-treated, was associated with enhanced expression of γENaC. Therefore, γENaC subunit appears to be critical for regulating the activity of ENaC under different culture conditions in mouse endometrial epithelium.

The importance of γENaC subunit may be further revealed by our recent demonstration of suppressed CFTR-mediated Cl^- secretion by enhanced expression of ENaC in mouse endometrium (Chan et al 2000b). In that study, only γENaC expression was found to be enhanced. Together with the present results, it suggests that γENaC may be involved in regulating ENaC functions, including upregulation of ENaC activity and an inhibitory effect on CFTR activity as previously observed (Chan et al 2000b). It should be noted that the C-terminal of γENaC subunit has a motif known to interact with a ubiquitous protein-ligase, mutations of which cause Liddle syndrome (Firsov et al 1996, Snyder et al 1995, Snyder 2000). Therefore, interaction between ENaC and CFTR may also be mediated by γENaC with a potential site for protein-protein interaction.

In summary, the present study has demonstrated differential regulation of ENaC

subunits in mouse endometrial epithelial cells. Only γENaC subunit mRNA, but not other subunits, was found to be upregulated upon hormone treatment or extracellular matrix affecting cell differentiation. Therefore, γENaC subunit appears to be important in regulating ENaC activity and thus the rate of Na^+ absorption across the mouse endometrium. γENaC subunit may also participate in the interaction between CFTR and ENaC thereby determining the delicate balance between Cl^- secretion and Na^+ absorption in the endometrium.

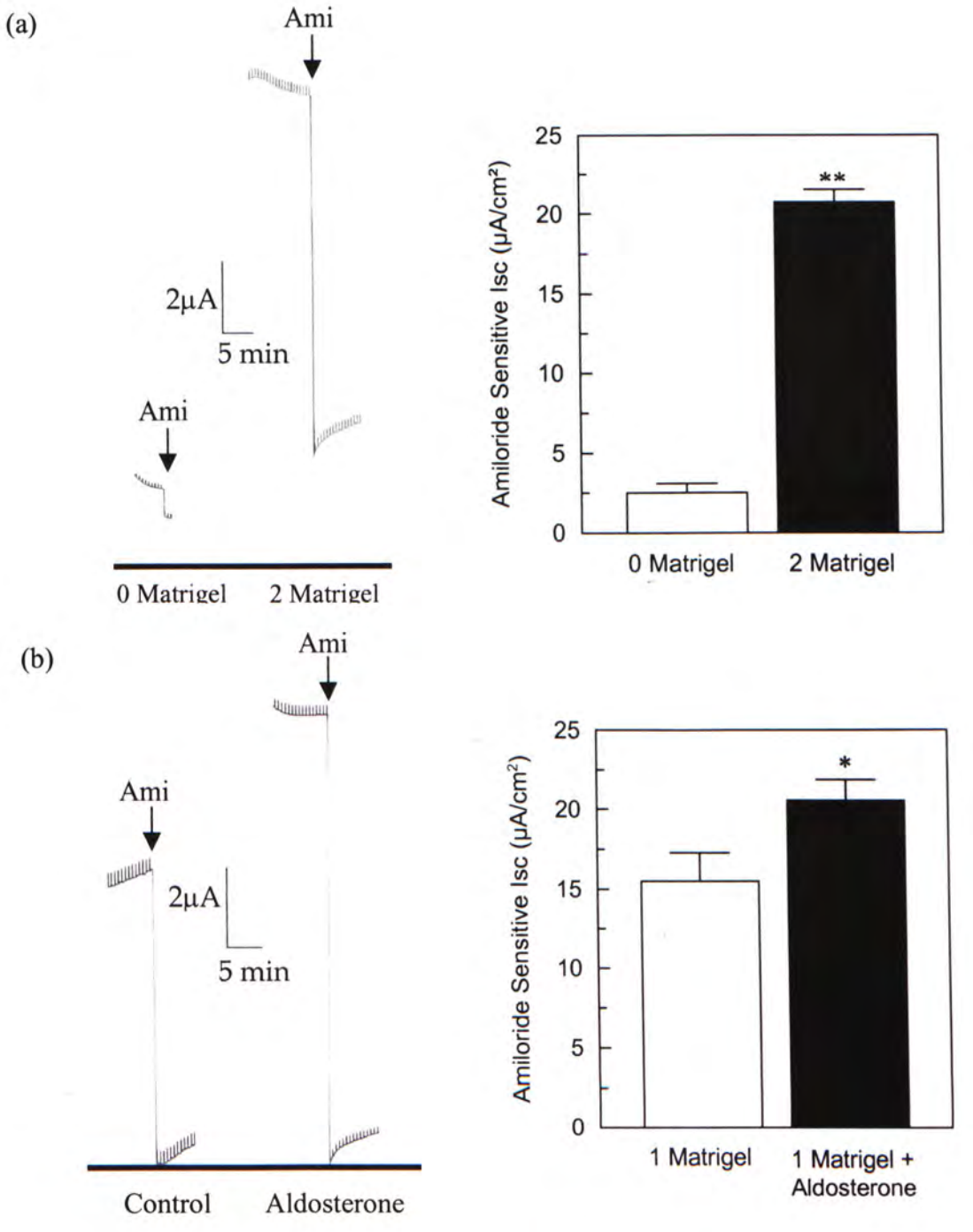


Fig 3.4.1 Effect of culture conditions on ENaC activity. (a) Representative I_{SC} recordings of amiloride-sensitive I_{SC} obtained from non-Matrigel-treated (0 Matrigel, n=27) and double-Matrigel-treated (2 Matrigel, n=15) cultures with statistical results (**P<0.001). (b) Representative I_{SC} recordings of amiloride-sensitive I_{SC} obtained from single- Matrigel-treated without (control, n=3) and with aldosterone (1 μM, n=10) treatment (*P<0.05). Concentration of amiloride was 10 μM.

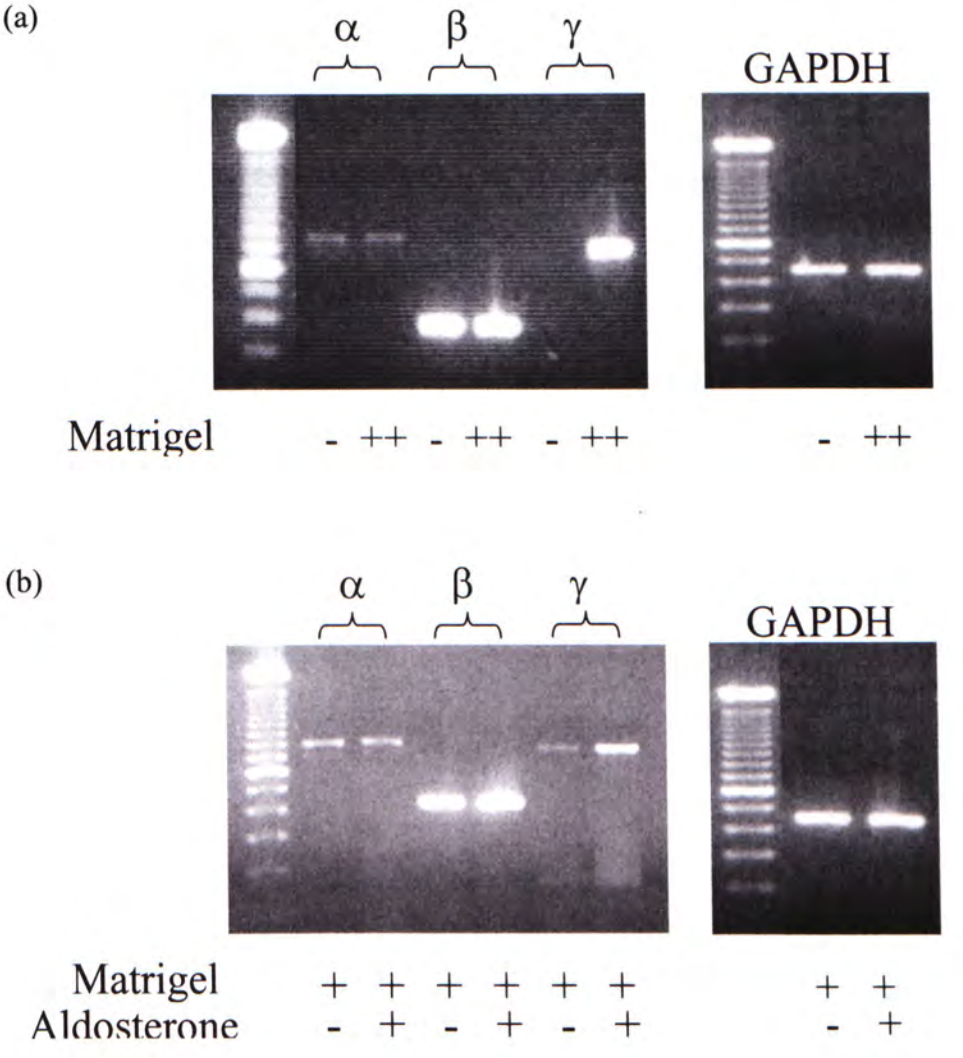
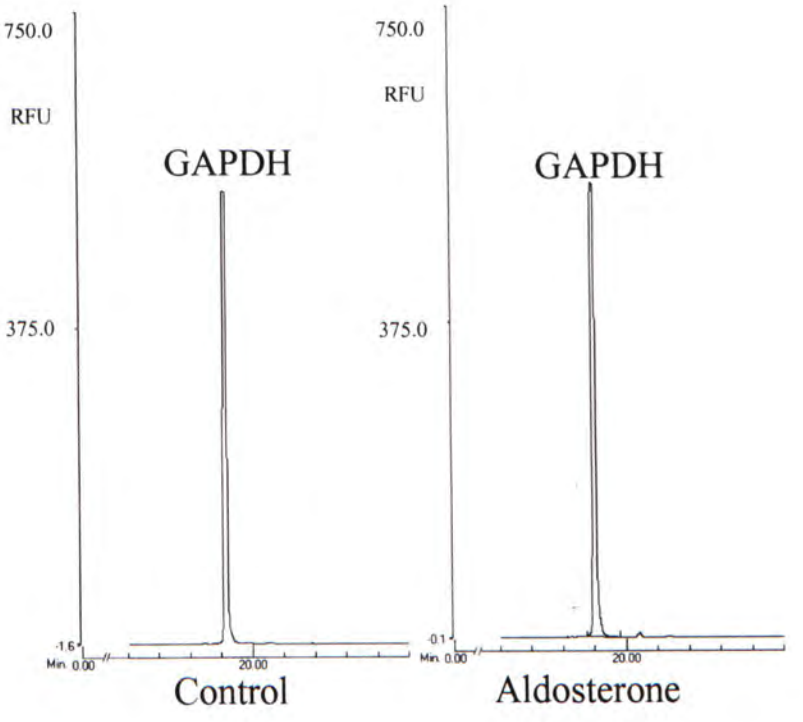
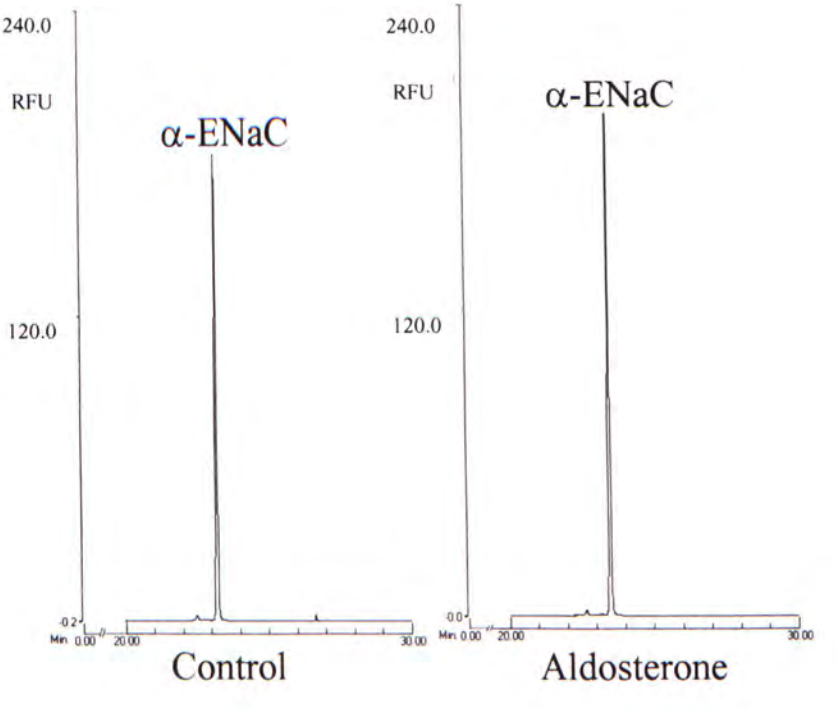


Fig 3.4.2 Semi-quantitative RT-PCR analysis demonstrating differential expression of ENaC subunits. (a) Comparison of subunits expression in non-Matrigel- treated(-) and double-Matrigel-treated (++) cultures. (b) Comparison of subunits expression in single-Matrigel-treated (+) cultures without (-) and with (+) aldosterone treatment. GAPDH was used as an internal standard. The experiment were repeated 3 times with similar results.

(a)


750.0
RFU
375.0
GAPDH
-1.6
Min. 0.00
20.00
Control
750.0
RFU
375.0
GAPDH
-0.1
Min. 0.00
20.00
Aldosterone

(b)


240.0
RFU
120.0
α-ENaC
-0.2
Min. 0.00
20.00
30.00
Control
240.0
RFU
120.0
α-ENaC
0.0
Min. 0.00
20.00
30.00
Aldosterone

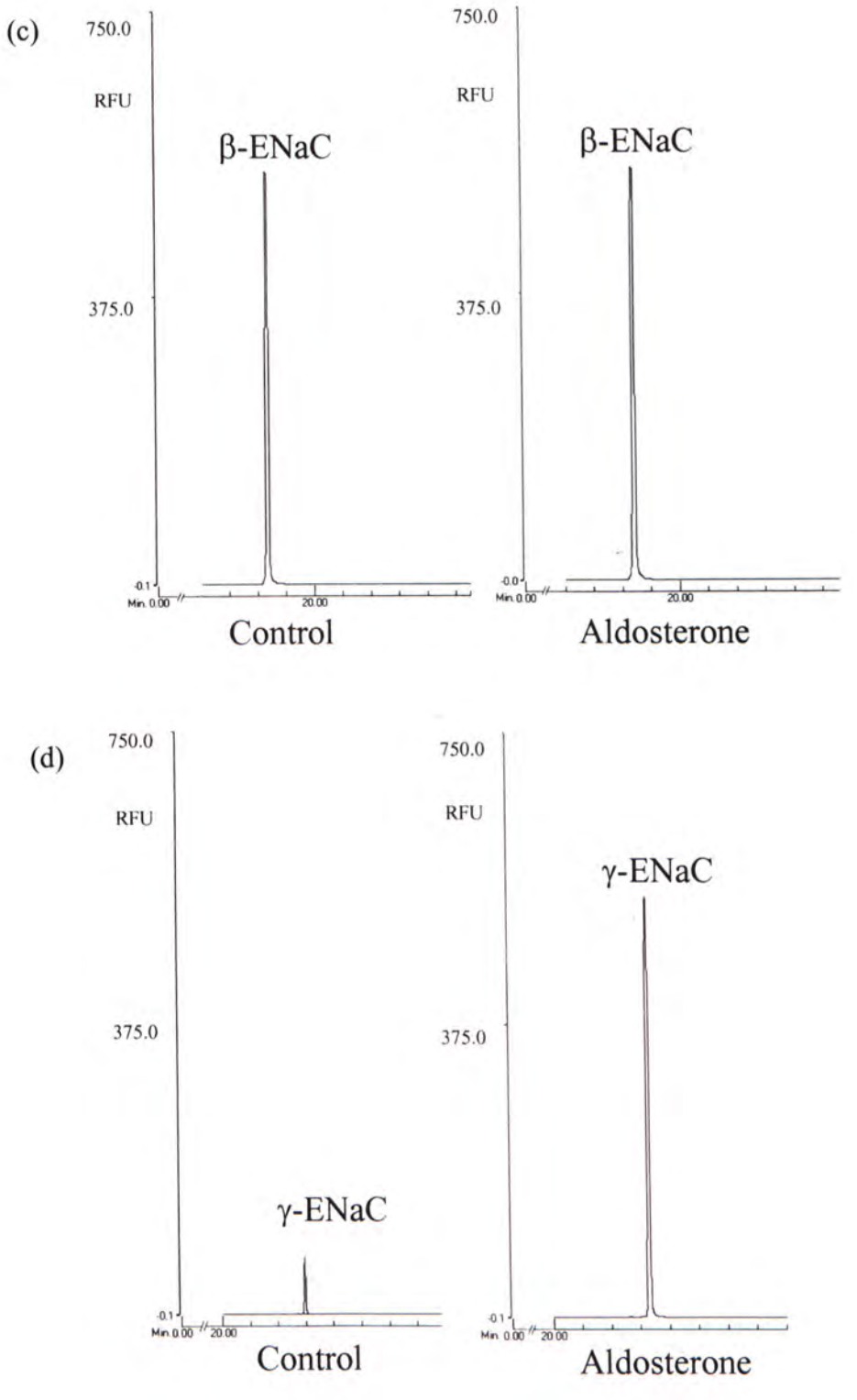


Fig 3.4.3 CE-LIF measurement of RT-PCR products of ENaC subunits. Comparison of the amount of RT-PCR products in control and aldosterone-treated cells: (a) GAPDH; (b) αENaC; (c) βENaC; (d) γENaC. Measurement were made 3 times and similar results were obtained.

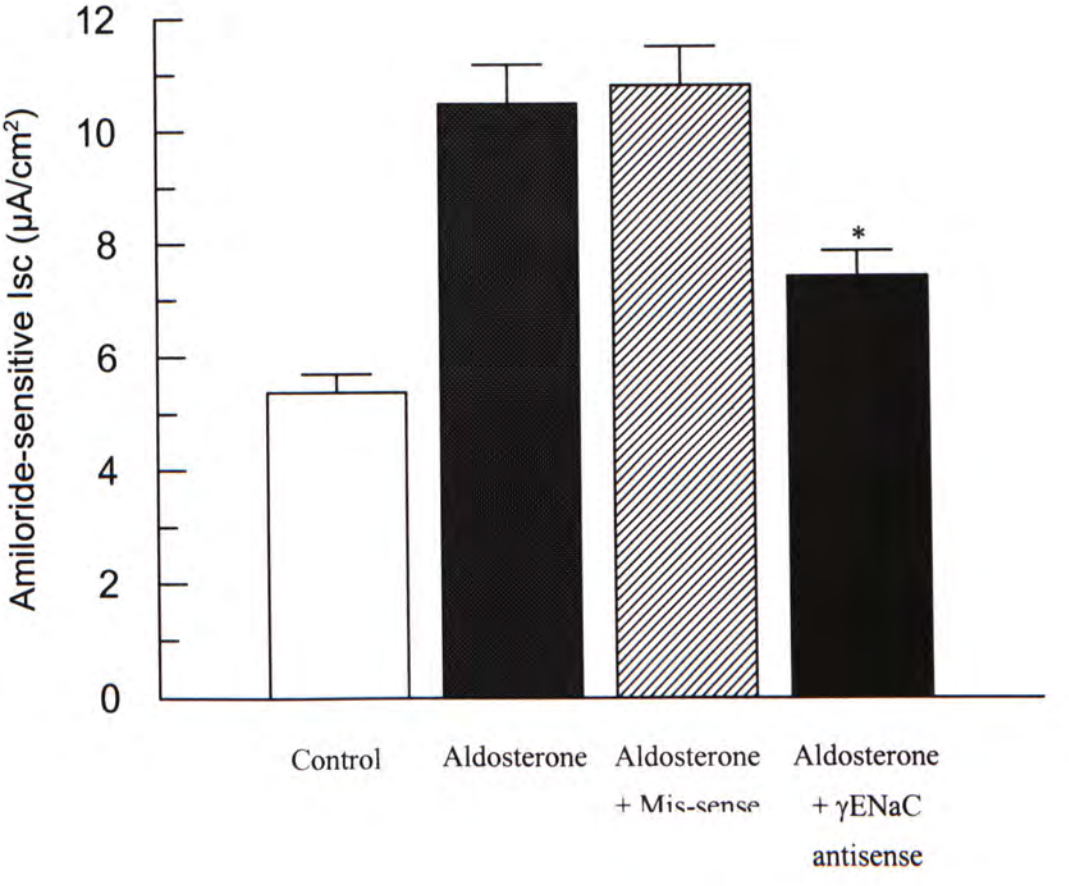


Fig 3.4.4 Effect of γENaC antisense on aldosterone-enhanced amiloride-sensitive I_{SC}. Mean currents ± S.E.M. obtained under different conditions were plotted (n=6, *P<0.05). Antisense (specific oligo nucleotide: TAC AGA TAC TCT CAG TTA AAA GAC) or mis-sense (specific oligo nucleotide: GTT TTC TTC TGC TTG GTC CA) (5 μg/ml) and aldosterone (1 μM) were added to cultures 24 hours prior to experiments.

Chapter 4: General Discussion

The endometrial epithelium plays an indispensable role in creating a suitable uterine microenvironment for sperm movement and capacitation, blastocyst development and implantation. It is believed that the uterine fluid functions to suspend, maintain and stimulate spermatozoa and the blastocyst during their transport process along the uterine lumen. Deviations from the normal compositions of uterine fluid have been found to be responsible for the reduced rate of fertilization seen in women (Harper 1994).

The absorptive and secretory activities of the endometrial epithelium largely determine the formation of optimal uterine microenvironment for various reproductive events. Previous studies using the short-circuit current (I_{SC}) technique have revealed the involvement of amiloride-sensitive Na^+ channels, mediated by epithelial sodium channel (ENaC) and cAMP-activated Cl^- channels, presumably mediated by cystic fibrosis transmembrane conductance regulator (CFTR) in the absorptive and secretory process of the mouse endometrial epithelium (Chan et al 2000b, Chan et al 2000a). These absorptive and secretory activities are believed to be finely modulated by ovarian hormones (estrogen and progesterone). However, the roles of ovarian hormones (estrogen and progesterone) in regulating CFTR and ENaC in mouse endometrial epithelium are poorly understood.

The endometrial epithelium is among the various epithelia expressing CFTR, which is a cAMP-dependent Cl^- channel. The relationship between expression of CFTR and ENaC, and uterine fluid volume has been found tightly coupled to estrus cycle and influenced by estrogen and progesterone, suggesting an important role of CFTR and ENaC in the normal uterine functions. The present studies showed that 17β-estradiol and progesterone treatment would increase CFTR and ENaC expression respectively, indicating that CFTR and ENaC are differentially regulated by estrogen and progesterone both *in vivo* and *in vitro*. Differential expression of CFTR and ENaC throughout the estrus cycle also indicates the importance of uterine fluid regulation during estrus cycle. Hormonal regulation of the uterine fluid volume has been demonstrated in rat and mice, indicating that estrogen treatment would increase uterine fluid volume while progesterone treatment would reduce the uterine fluid volume (Casslen 1986). Moreover, the importance of CFTR in endometrial function has also been emphasized by the previously demonstrated interaction between CFTR and ENaC in the endometrial epithelium (Chan et al 2000b). The present study is the first to provide molecular basis for the observed changes in uterine fluid volume during estrus cycle in terms of expression of CFTR and ENaC in the mouse uterus.

In addition to the role of ovarian hormone in regulating mRNA expression of CFTR and ENaC, the present study has demonstrated the reciprocal expression relationship between mRNA expression of CFTR and ENaC under different stages of estrus cycle. The presently demonstrated reciprocal expression pattern for CFTR and

ENaC during estrus cycle may provide an explanation as to how the endometrial epithelium can maintain an optimal fluid micro-environment for various reproductive events under the influence of ovarian hormones. It may also provide a regulatory mechanism that the endometrium can be conditioned to be overall absorptive by suppression of CFTR expression via enhancing ENaC expression in response to changes in hormonal levels. The interaction between CFTR and ENaC may also determine the delicate balance between Cl^- secretion and Na^+ absorption, thereby creating an optimal uterine fluid environment for sperm motility, embryo implantation and development. Taken together, the present finding of CFTR and ENaC under the influence of ovarian hormones (estrogen and progesterone) and differential expression in estrus cycle may provide a physiological basis for the reduced rate of fertility observed in CF women. However, the precise role of CFTR and the three subunits of ENaC in various reproductive events remains to be elucidated.

We should note that there is discrepancy in the present results obtained *in vivo* and *in vitro* on the CFTR expression, such as the decreased CFTR mRNA expression in 48-hour progesterone injection treatment using RT-PCR technique, and the increases forskolin-induced I_{SC}, presumably mediated by CFTR, in aldosterone treatment. Aldosterone and progesterone are well-known for their effect on stimulating ENaC expression, and our results were consistent both *in vivo* and *in vitro*. However, their effect on CFTR expression was puzzling. The regulation of CFTR by aldosterone may be more complicated *in vivo* than *in vitro*. Culture condition may be one of the factors

that affect the experimental results. Future studies on detail mechanism by which these ion channels are regulated require further investigation.

One of the important findings of the present study is the role of γENaC in determining uterine ENaC activity. The present study has demonstrated the functional role of ENaC activity in the mouse endometrial epithelium under different culture conditions (double Matrigel and aldosterone treatment) measured by I_{SC}. However, the precise role of each ENaC subunit in regulating ENaC activity was poorly understood. Differential expression of different ENaC subunits in response to aldosterone has been observed in different tissues with marked increase in αENaC mRNA in mouse and rat kidney (Loffing et al 2000, MacDonald et al 2000, Masilamani et al 1999) but enhanced β and γENaC mRNA expression in rat distal colon (Epple et al 2000, Lingueglia et al 1994, Renard et al 1995). These differences could be due to tissue-specific transcription efficiency, and, no firm conclusion can be drawn as to which specific subunit is responsible for aldosterone-enhanced ENaC activity since no direct demonstration has been made linking transcriptional changes directly to functional variations in ENaC activity. The present study coupling ENaC expression and amiloride-sensitive I_{SC} indicated that γENaC subunit appears to be critical for regulating the activity of ENaC under different culture conditions in mouse endometrial epithelium.

When the body Na^+ homeostasis is disturbed such as loading with a low Na^+ diet for a long period, the circulating aldosterone is expected to be elevated. The presently

demonstrated the expression pattern of uterine ENaC and CFTR of mice fed with a low Na^+ diet during estrus cycle might provide a functional role of each subunit of ENaC and CFTR in response to disturbance in Na^+ homeostasis. Under such condition, the characteristic cyclic expression of CFTR in uterus was altered in order to maintain Na^+ homeostasis. Therefore the secretory event in uterus was suppressed in order to conserve Na^+ in uterine fluid and prevent pregnancy. This may be to ensure fertilization under a healthy body condition. Therefore uterine CFTR and ENaC may be tightly regulated by the hormones affecting electrolyte and fluid homeostasis.

In conclusion, the present study has provided a molecular basis for the uterine fluid formation during estrus cycle in terms of expression of CFTR and ENaC in the mouse uterus. The present study has demonstrated the reciprocal expression between mRNA expression of CFTR and ENaC under different stage of estrus cycle. In addition, the present study has also demonstrated the involvement of uterine CFTR and ENaC in maintaining Na^+ homeostasis. It is hoped that the result of the present study may provide more understandings on the regulatory mechanisms for electrolyte transport across the endometrial epithelium, and may eventually provide grounds for the development of contraceptives and new treatment for infertility.

Appendix A RNA Isolation

Mix endometrial epithelial cells/uterus tissues with TRIZOL reagent

(10^6 cells per 1 ml)

⇩

Homogenize

⇩

Mix with 0.2 ml choloform

⇩

Shake for 15 seconds

⇩

Centrifuge at 14,000 x *g* at 4°C for 10 minutes

⇩

Transfer the upper aqueous layer to a 1.5 ml microcentrifuge tube

⇩

Add 0.5 ml isopropanol

⇩

Incubate at 4°C for 10 minutes

⇩

Centrifuge at 14,000 x *g* at 4°C for 10 minutes

⇩

Discard the supernatant

⇩

Wash with 1 ml 70% cold ethanol for wash

⇩

Air-dry for 3 – 5 minutes

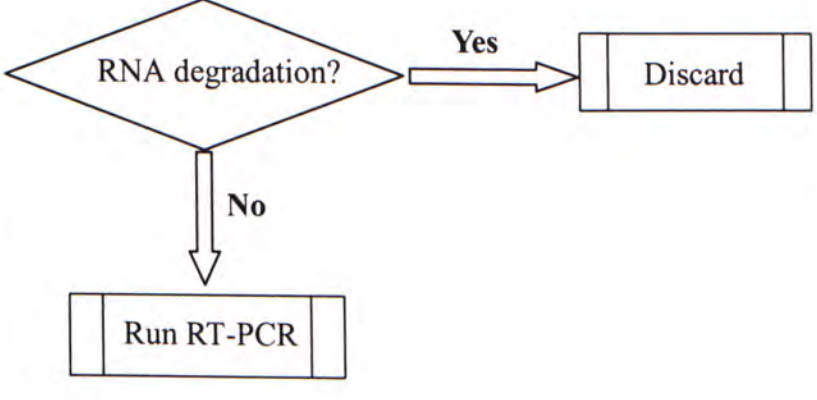
⇩

Dissolve in 20 µl DEPC-treated H_2O

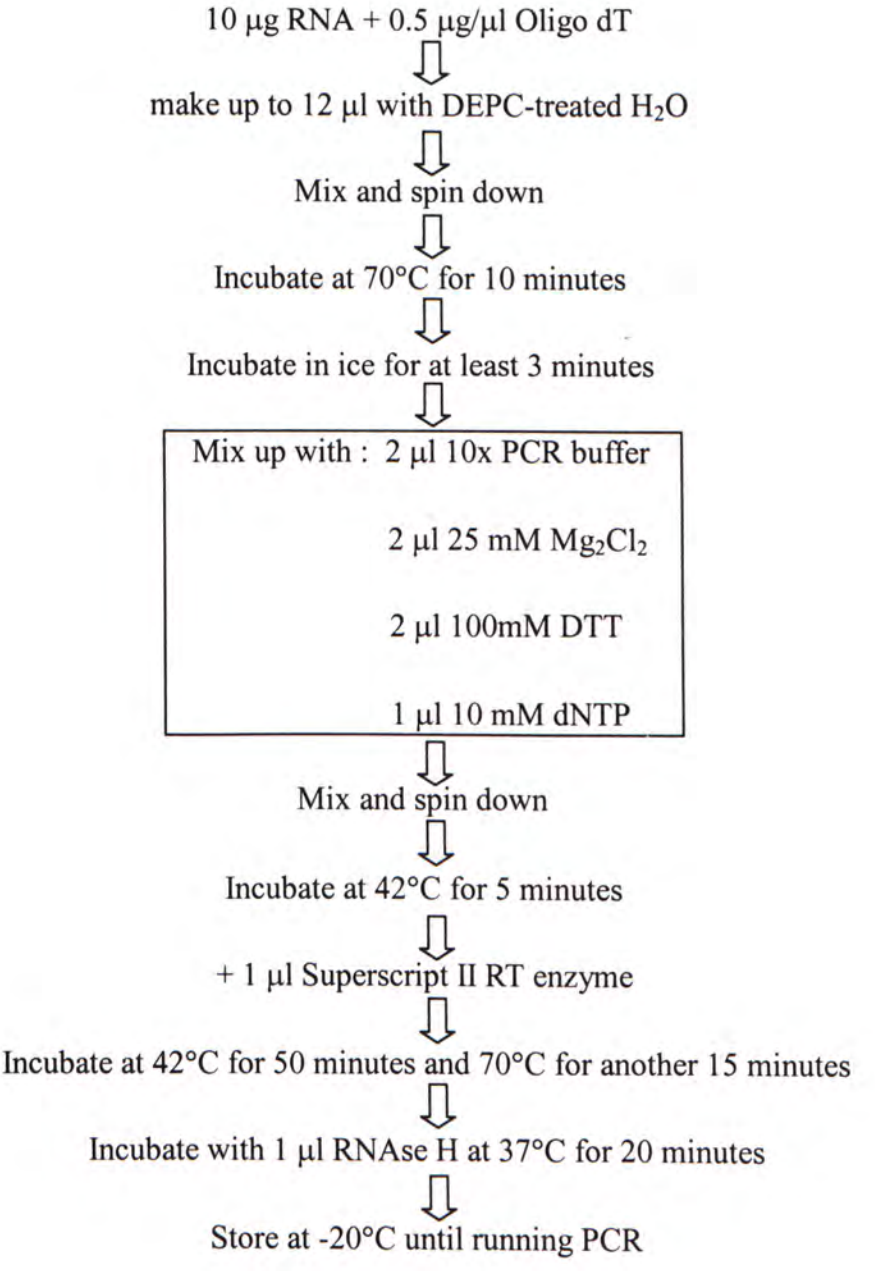
⇩

RNA gel electrophoresis

⇩



Appendix B Reverse-Transcription (RT)



Appendix C Polymerase Chain Reaction (PCR)

General PCR

Component	Concentration	Volume (μl)
10 x PCR buffer	1 x	2.5
10 mM dNTP mixture	0.2 mM	0.5
Target Primer (sense)	250 nM	2.5
Target Primer (antisense)	250 nM	2.5
First strand cDNA template (from RT)	-	1
DEPC-treated water	-	15.8
*Taq DNA polymerase (5U/1μl)	1U	0.2
Final volume of the working PCR tube is 25 μl		

Semi-quantitative RT-PCR

Component	Concentration	Volume (μl)
10 x PCR buffer	1 x	2.5
10 mM dNTP mixture	0.2 mM	0.5
Target Primer (sense)	250 nM	2.5
Target Primer (antisense)	250 nM	2.5
GAPDH Primer (sense)	250 nM	2.5
GAPDH Primer (antisense)	250 nM	2.5
First strand cDNA template (from RT)	-	1
DEPC-treated water	-	10.8
*Taq DNA polymerase (5U/1μl)	1U	0.2
Final volume of the working PCR tube is 25 μl		

* Taq was added before PCR reaction start.

Appendix D **Sequences and Conditions of All Primers**

GAPDH	Size = 451 bp	Annealing temp = 53 °C	
5' - ACC ACA GTC CAT GCC ATG AC - 3' 3' - TCC ACC ACC CTG TTG CTG TA - 5'			
GAPDH	Size = 340 bp	Annealing temp = 53 °C	
5' - GAC CAC AGT CCA TGC CAT CAC TGC - 3' 3' - GCT GTT GAA GTC GCA GGA GAC AAC - 5'			
CFTR	Size = 481 bp	Annealing temp = 58 °C	Cycle = 30
5' - CAT CTT TGG TGT TTC CTA TGA TG - 3' 3' - GTA AGG TCT CAG TTA GAA TTG AA - 5'			
α-ENaC	Size = 790 bp	Annealing temp = 62 °C	Cycle = 32
5' - TCA CTT CAG CAC ATC TTC CAC AGC TGC - 3' 3' - GTA TCT GCC TAC CTG GTC CAA GTG GGA - 5'			
β-ENaC	Size = 420 bp	Annealing temp = 53 °C	Cycle = 30
5' - CCC CAC CCA GCA ACT AGT GAA CTC AAA - 3' 3' - AAA GCA CGT GTT CCC CTT TCA AGA CTT - 5'			
γ-ENaC	Size = 724 bp	Annealing temp = 59 °C	Cycle = 30
5' - GAC TCT CTT CCT GAC ACA AAT GGT CCT - 3' 3' - ACA CAC ATT CTC ACA CAT ACA CAT ACT - 5'			

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